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Central Immune Tolerance of T and B Cells in Patients With Idiopathic Hypoparathyroidism, T1D, and Autoimmune Thyroiditis

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Context: Pathogenesis of idiopathic hypoparathyroidism (IH) is under investigation. Abnormalities in central immune tolerance have yet not been investigated in this condition. T-cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs), formed during receptor gene rearrangements, are tools to assess central T- and B-cell output.

Objective: We assessed the number of circulating TRECs and KRECs in patients with IH, autoimmune type 1 diabetes (T1D), and autoimmune thyroiditis (ATs) and healthy controls (HCs).

Design: Comparative case-control at tertiary care center.

Subjects and Methods: Absolute and relative TRECs and KRECs were measured in DNA extracted from whole blood of patients with IH (n = 181, 22 of whom were reassessed after a decade of follow-up) and T1D (n = 133), AT (n = 53), and HC (n = 135) using a quantitative real-time PCR/TaqMan® probe technique.

Results: Absolute and relative means of TRECs and KRECs in IH were comparable to HCs, and no differences were found between IH with and without calcium-sensing receptor antibodies or class I *HLA-A*26:01* association. TRECs and KRECs did not change after a decade of follow-up. T1D had significantly higher absolute TRECs than IH, AT, and HCs, whereas AT patients showed lower TRECs and the highest KRECs; these levels showed no noteworthy correlation with thyroid dysfunctions.

Conclusion: Patients with IH showed TRECs and KRECs comparable to HCs, indicating an intact mechanism of T- and B-cell central immune tolerance. Interestingly, absolute TRECs were significantly higher in T1D than HCs, suggesting impaired central immune tolerance in T1D.

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Freeform/Key Words: T-cell receptor excision circles, kappa-deleting recombination excision circles, autoimmunity, CaSRAb, idiopathic hypoparathyroidism

Abbreviations: AIIMS, All India Institute of Medical Sciences; AT, autoimmune thyroiditis; BMI, body mass index; CaSR, calcium-sensing receptor; CaSRAb, calcium-sensing receptor antibody; Ct, cycle threshold; *GCM2*, glial cell missing 2; HC, healthy control; IH, idiopathic hypoparathyroidism; IQR, interquartile range; KREC, kappa-deleting recombination excision circle; q-PCR, quantitative real-time-PCR; T1D, type 1 diabetes; TPOAb, thyroid peroxidase antibody; TREC, T-cell receptor excision circle.

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The characteristic clinical features of idiopathic hypoparathyroidism (IH) [*i.e.*, tetany, convulsions, cataract, intracranial calcification, and subnormal PTH] have been known for many decades [1]. However, the pathogenesis of IH is not very clear, and, therefore, the disease is also referred to as isolated, sporadic, and acquired hypoparathyroidism [2, 3]. Recent studies suggest a heterogeneous etiology of IH involving either parathyroid autoimmunity or genetic mutations in *PTH*, calcium-sensing receptor (*CaSR*), glial cell missing 2 (*GCM2*), and autoimmune polyendocrinopathy candidiasis-ectodermal dystrophy-autoimmune regulator (*APECED-AIRE*) genes [4–8]. Protein tyrosine phosphatase nonreceptor (*PTPN*) or cytotoxic T lymphocyte antigen-4 (*CTLA4*) polymorphisms, involved in defective peripheral immune tolerance, were not associated with IH [9, 10], whereas autoantibodies against parathyroid tissue using indirect immunofluorescence have been found in patients with IH [11]. Subsequent identification of specific CaSR autoantibodies (CaSRAbs) in subsets of patients with IH suggested a role of humoral autoimmunity in the pathogenesis of this disease [4, 5, 12]. Modest prevalence of CaSRAbs in IH (~12.0%) could be due to either a limited role of B-cell autoimmunity or the disappearance of these autoantibodies with increasing duration of disease and loss of parathyroid tissue [5]. A larger role of B-cell/humoral autoimmunity in the initial stages of IH cannot be ruled out. In addition, our earlier studies also indicated a role of major histocompatibility complex class I, *HLA-A*26:01*–restricted cytotoxic $CD8^+$ T cells in autoimmune responses in IH indicated by exaggerated interferon-γ response in the peripheral blood mononuclear cells stimulated with *HLA-A*26:01*–restricted peptides of CaSR in patients with IH [13, 14].

Impaired central immune tolerance has recently been identified in autoimmune disorders [*i.e.*, type 1 diabetes (T1D), Graves disease, and immune thrombocytopenic purpura] [15–17]. The normal process of central immune tolerance involves a negative selection and autodeletion of T and B cells from thymus and bone marrow, respectively. The education in these central lymphoid organs involves generation of diverse repertoires of T- and B-cell receptors by random recombination of variable, diversity, and joining [V(D)J] segments of DNA. The segments excised during recombination [*i.e.*, T-cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs)] remain in the matured T and B cells as circular DNA molecules. The cells expressing receptors for autoantigens are normally deleted, along with their TRECs or KRECs, in the central lymphoid organs following contact with local epithelial cells expressing corresponding antigens. In impaired central immune tolerance, a large number of autoreactive T and B cells, along with their respective TRECs and KRECs, escape into circulation and home in various organs predisposing to autoimmunity. Because the circular excised DNA remains stable in the cells and does not replicate during subsequent divisions of T and B lymphocytes, the TREC and KREC assay is a sensitive tool to measure T- and B-cell output from thymus and bone marrow, respectively [18–20]. Furthermore, any abnormal increase in concentration in any of these molecules would serve as an indirect marker of heightened escape from central immune tolerance.

To our knowledge, there are no previous studies investigating the levels of TRECs and KRECs in IH. In this study, we report the values of circulating TRECs and KRECs in patients with IH compared with those with T1D, patients with autoimmune thyroiditis (AT), and subjects used as healthy controls (HCs) to understand the relative role of T- and B-cell autoimmunity in IH.

1. Materials and Methods

A. Patients and Controls

Patients with IH (n = 181) were part of a group of 226 patients managed by the investigators since 1998 in the endocrine clinics of the All India Institute of Medical Sciences (AIIMS) in Delhi India [21–27]; most of them have been part of our earlier studies [22–26]. Patients with T1D (n = 133) and AT (n = 53), taken as prototype autoimmune endocrine disorders for comparison, were recruited at the Diabetes of Young and General Endocrine Clinics of

AIIMS, respectively. T1D was classified according to the American Diabetes Association, and only those with firm biochemical or clinical evidence of autoimmunity (*i.e.*, glutamic acid decarboxylase-65 autoantibodies and/or coexistent thyroid or celiac autoimmunity) were included in the study. The diagnosis of AT was based on serum thyroid peroxidase antibody (TPOAb) titers >34.0 IU/mL (n = 52) or from cytology of the thyroid gland (n = 1). HCs (n = 135) were subjects with normal TPOAb titers (<34 IU/mL) who were age matched (within ±2 years) with IH. They were selected from a cohort of 655 individuals enrolled for our earlier study related to vitamin D and thyroid autoimmunity [27]. After obtaining written consent, the genomic DNA was extracted from the whole blood cells and stored at −80°C. Only those patients with IH with sufficient quantity of stored DNA for performing TREC and KREC assay were included in the current study. The study was carried out in accordance with the tenets of the Declaration of Helsinki. The ethics committee of AIIMS approved the study protocol (IEC-275/06.05.2016, RP-24/2016), including the use of the stored DNA [4, 5].

The TREC and KREC content was measured by quantitative real-time-PCR (q-PCR) using TaqMan® probes. The precise quantification of DNA in the samples and the standardization of q-PCR required meticulous attention to each step, starting with the attempt to avoid any form of aerosol contamination. The DNA of all study subjects was quantified in batches before each q-PCR using an ultraviolet spectrophotometer (GeneQuant 1300; GE Healthcare Life Science, Cambridge, United Kingdom) and then diluted to10 ng/μL. The precision of DNA quantification was assessed by repeated measurements of two DNA samples from patients with T1D with concentrations of 1429 ng/μL and 747 ng/μL, respectively. Both of these samples were put at an interval of every 15 samples during DNA quantification. The interassay and intra-assay coefficients of variation for the two DNA quantifications were 5.6% and 1.7% and 4.9% and 2.7%, respectively.

The primer sequences for TREC assay were designed by Primer-3 software (National Center for Biotechnology Information, Bethesda, MD), whereas the probe was described earlier by Douek *et al.* [28]. Primers and probe for KRECs were described earlier by Sottini *et al.* [29] and those for albumin gene by Strawa *et al.* [16]. The sequences of the primers, fluorescent probes, and amplicon sizes are given in Table 1. The 25.0-μL PCR reaction contained 12.5 μL iTaq™ supermix (Bio-Rad Laboratories, Hercules, CA), 50.0 ng of genomic DNA in 5 μL of deionized water, 1 μL each of reverse and forward primers (3.0 μM each), and TaqMan® probes for TRECs and KRECs (5.0 μM each), respectively diluted in 1.5 μL nuclease-free water. The q-PCR reactions for TRECs and KRECs were carried out in duplicate in 96-well plates. Reference albumin gene was amplified along with TREC and KREC molecules under the same conditions, but in separate wells of the PCR plate. Each plate included samples of IH, T1D, AT, and HC in a ratio of 2:2:1:2. q-PCR was performed using a CFX-96 thermocycler (Bio-Rad Laboratories) with initial heating at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds and annealing-elongation at 60°C for 1 minute.

In this study, the TREC and KREC levels are given as absolute (copies/50 ng of genomic DNA) and relative (copies/10^3 blood cells) values. The absolute copies of TRECs and KRECs were determined using standard curves prepared by diluting pCR®2.1-TOPO plasmid (Thermo Fisher Scientific, Waltham, MA), which includes TREC and KREC inserts. The

Table 1. Primer and Probe Sequences Used During q-PCR for TRECs, KRECs, and Albumin Gene

Targets	Forward and Reverse Primers	Probe	Product (bp)
TREC	5′-TGCCACATCCCTTTCAACCA-3′, 5′-CAGGTGCCTATGCATCACCG-3′	5′-FAM-ACACCTCTGGTTTTTGTAAA GGTGCCCACT-BHQ1-3′	96
KREC	5′-TCCCTTAGTGGCATTATTTGTATCACT-3′, 5′-AGGAGCCAGCTCTTACCCTAGAGT-3′	5′-Cyc-TCTGCACGGGCAGCAGGT TGG-BHQ2-3′	90
Albumin	5′-GCTGTCATCTCTTGTGGGCTGT-3′, 5′-ACTCATGGGAGCTGCTGGTTC-3′	5′-HEX-GGAGAGATTTGTGTGGGCAT GACAGG-BHQ1-3′	138

Abbreviation: bp, base pair.

quantity of plasmid to prepare a bulk of first standard curve point at 10^6 copies of TREC/ KREC plasmid/ 5.0 μL of deionized water was calculated according to Sottini *et al.* [30]. The subsequent standard points were prepared fresh before each PCR by exponential dilution ranging from 10^5 to 10^1 plasmid copies each in 5.0 μL deionized water. The six dilution points were put in triplicate in the q-PCR assay to generate the standard curve. As previously reported [16], three standard curves were put, during the whole study, at the beginning, in the middle, and at the end of the q-PCR assay and are used to generate a composite standard curve and a regression equation for absolute and relative quantification. The interassay and intra-assay coefficients of variation for TRECs and KRECs for absolute copies were 26.1% and 21.0% and 6.2% and 12.6%, respectively.

The relative TREC and KREC copies were expressed per 10^3 blood cells and calculated starting from a standard curve of genomic DNA, pooled from five HCs, for albumin gene quantification. As noted previously, three albumin gene standard curves were set up to generate a composite curve. The DNA dilutions used in each standard curve were 500 ng/well, 250 ng/well, 100 ng/well, 50 ng/well, 25 ng/well, 5 ng/well, and 1 ng/well. The albumin gene copies present in each sample were calculated using the corresponding genomic DNA amount, obtained through the standard curve, and assuming that a diploid cell contains an average of 6.6×10^{-3} ng of genomic DNA. Then, the relative TREC or KREC copies per 10^3 cells were calculated using the formula: ($2 \times$ copy number of TRECs or KRECs/copy number of albumin $\times 10^3$). The efficiency of TREC, KREC, and albumin amplification, checked through the slope of the standard curves [efficiency = $10^{(-1/\text{Slope})} - 1$], varied from 96% to 99% for TREC, 95% to 97% for KREC, and 97% to 99% for albumin assays (Fig. 1).

The interassay and intra-assay coefficients of variation for cycle threshold (Ct) values for TRECs, KRECs, and albumin were all $<1\%$; those for relative copies of TRECs and KRECs were 28.6% and 22.3% and 5.1% and 17.1%, respectively. Two approaches were used to address the possible effect of prolonged duration of storage of DNA in IH. These included: (1) relative quantification of circulating TREC and KREC molecules in relation to albumin gene; and (2) stratifying the whole group of patients with IH into three subsets based on the year when patient DNA was extracted and stored. Subset I included patients with IH who presented to us during the years 1998 to 2004 (n = 61), subset II were patients presenting from 2005 to 2009 (n = 53), and subset III had patients who presented from 2010 until 2016 (n = 67). Thus, the duration of DNA storage in these three subsets ranged from 12 to 18 years, 7 to 11 years, and 1 to 6 years, respectively. To assess the possible change in TRECs and KRECs from baseline during progression of the disease, the genomic DNA was isolated again in a subset of patients with IH (n = 22) during their follow-up in endocrine clinics. The mean interval at which DNA was re-extracted from these 22 patients was 9.6 ± 4.4 years [median and interquartile range (IQR) 10.0 (6.0 to 12.3)].

B. Statistical Analysis

Data are shown as mean, SD, and frequency (in percentages). Analysis of variance with Bonferroni correction was used to compare various indices in the IH, T1D, AT, and HC. A

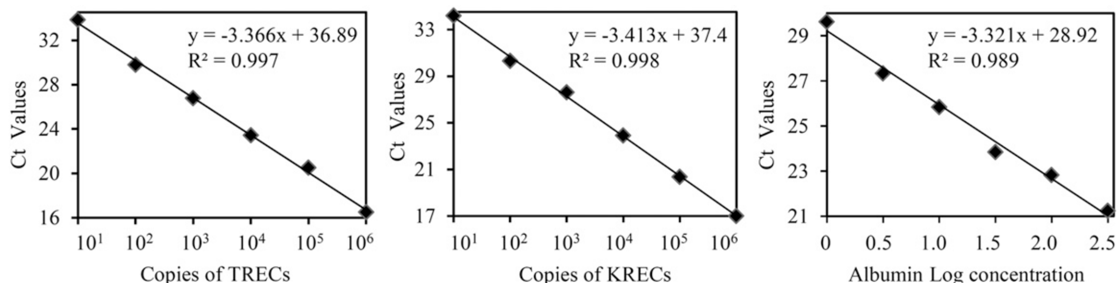

Figure 1. Standard curves showing log regression for TRECs, KRECs, and albumin gene concentration and Ct. R^2, regression coefficient; y, Ct values.

two-tailed P value <0.05 was considered significant. Pearson correlation analysis was used to assess the relationship between duration of illness and circulating TRECs and KRECs. Wilcoxon signed-rank test was used to compare the mean TREC and KREC copies in the 22 patients at initial presentation and again after a decade of follow-up.

2. Results

A. Patients' Characteristics

The clinical features at diagnosis of IH, including history of tetany (90.1%), convulsion (60.8%), frequency of cataract (44.8%), basal ganglia calcification (86.7%), mean serum calcium (5.5 ± 1.0 mg/dL), inorganic phosphorus (6.9 ± 1.5 mg/dL), and median (IQR) of intact PTH [6.02 (2.96 to 10.68) pg/mL], were similar to those reported earlier [20–25]. Ten percent of the patients with IH (13 out of 129 subjects tested) had serum CaSRAbs (>3.0 SD score of HC), and 8.5% (15 out of 176) had R110W mutation of the *GCM2* gene [4, 7]. Coexistent thyroid autoimmunity in IH (TPOAb >34.0 IU and/or TSH >10.0 μIU/mL) was 16.67%. None of the patients with IH had T1D. Among the 133 subjects with T1D, 76.6% had glutamic acid decarboxylase-65 autoantibodies (85 out of 114 tested), and 58.8% had TPOAb positivity (58 out of 97). Sixty-five of the 133 patients with T1D (48.8%) were on thyroxin replacement. The mean TPOAb titer in the AT group was 355 ± 233 IU/mL. Fifty-one of the patients were on thyroxin replacement. Circulating TSH was >10.0 μU/mL in 21 patients with AT. None of patients in the AT group had coexistent T1D or IH.

Table 2 gives the demographic characteristics of the four study groups. Although the mean age of IH group was comparable to that of T1D and HC, it was significantly lower than that of the AT group. The mean body mass index (BMI) of the IH and T1D groups was comparable. However, BMI of both of these groups was significantly different from that of AT and HC. The numbers of females were significantly more in the AT group compared with other groups. There was a notable difference in the duration of illness among the T1D, AT, and IH groups.

B. TREC and KREC Quantification

The absolute and relative mean of TREC copies in the IH group were comparable to those of AT and HC, but significantly lower than those of T1D (Table 2). The absolute and relative TREC and KREC copies in IH T1D, AT, and HC is given in an online repository [31]. The T1D

Table 2. Clinical Characteristics and TREC and KREC Copies [Mean ± SD and Median (IQR)] in Patients With IH, T1D, and AT and HCs

					***P* Value**					
Parameters	**IH (n = 181)**	**T1D (n = 133)**	**AT (n = 53)**	**HC (n = 135)**	**IH vs T1D**	**IH vs AT**	**IH vs HC**	**T1D vs AT**	**T1D vs HC**	**AT vs HC**
Age, y	29.9 ± 13.2	30.7 ± 9.9	36.7 ± 13.8	32.4 ± 13.2	0.99	0.003	0.49	0.019	0.99	0.21
Male/female, number	91/90	55/78	12/41	79/56						
BMI, kg/m^2	20.8 ± 4.6	21.8 ± 5.3	24.5 ± 5.4	24.2 ± 4.2	0.39	<0.001	<0.001	0.003	<0.000	0.99
Duration of illness, y	7.1 ± 7.2	15.6 ± 7.8	3.0 ± 3.7	—	<0.001	0.001	—	<0.001	—	—
	5 (2–10)	15 (10–21)	2 (0.83–4)							
TREC copies/50 ng of genomic DNA	61 ± 44	75 ± 34	49 ± 33	59 ± 38	<0.001	0.13	0.99	<0.001	<0.001	0.07
	47 (32–76)	69 (51–97)	37 (28–51)	51 (35–68)						
TREC copies/10^3 blood cells	25 ± 21	35 ± 25	19 ± 16	32 ± 27	<0.001	0.07	0.11	<0.001	0.06	<0.001
	20 (11–32)	29 (18–43)	14 (9–20)	24 (12–40)						
KREC copies/50 ng of genomic DNA	74 ± 62	85 ± 77	104 ± 99	68 ± 56	0.30	0.14	0.99	0.99	0.11	0.06
	56 (34–91)	60 (41–99)	66 (37–122)	51 (34–80)						
KREC copies /10^3 blood cells	29 ± 24	33 ± 24	38 ± 35	31 ± 23	0.10	0. 47	0.99	0.99	0.83	0.99
	21 (14–34)	28 (16–41)	24 (16–48)	23 (15–39)						

group had the highest mean of TREC copies, which was significantly higher than those of AT and HC (Table 2). The absolute and relative mean of KREC copies in IH were comparable to those of T1D, AT, and HC. Interestingly, the AT group showed the highest mean of KRECs among the four study groups. The absolute median of TREC and KREC values [per 50 ng of genomic DNA (IQR)] were comparable at baseline and after a decade of follow-up in 22 patients with IH [19 (13 to 72) vs 24 (18 to 47) and 109 (66 to 209) vs 132 (93 to 161); $P = 0.36$ and $P = 0.88$, respectively]. Similarly, the relative median of TREC and KREC values [per 10^3 cells (IQR)] showed no significant difference between baseline and after follow-up ($P = 0.82$ and $P = 0.32$, respectively).

The absolute mean of KREC copies within AT group was comparable in patients with normal and raised serum TSH (115 ± 113 vs 98 ± 91 copies/50 ng of genomic DNA), respectively; $P = 0.82$). Similar results were obtained for TREC copies in the AT groups with normal and raised serum TSH.

In view of the considerable differences in sex, BMI, and duration of illness in the IH, T1D, and AT groups, a correlation analysis was performed between the previously mentioned indices with number of TREC and KREC copies. This was considered necessary to assess the confounding effect of these features in regression analysis. The absolute and relative mean of TREC and KREC copies were comparable between males and females when all of the four study groups were separately analyzed ($P > 0.05$ for all). There was no significant correlation between duration of illness in IH and absolute TREC and KREC copies ($P = 0.31$ and 0.42, respectively) and relative TREC and KREC copies ($P = 0.08$ for both). There was no significant correlation between TREC and KREC copies and duration of illness in the T1D and AT groups. Furthermore, there was no significant correlation between BMI and absolute or relative TREC and KREC values when all of the study groups were analyzed together or separately in IH or T1D.

The absolute TRECs and KRECs in subsets of IH samples (*i.e.*, those with DNA stored from 2010 to 2016, from 2005 to 2009, and from 1998 to 2004) showed no trend of decrease in their mean values with increasing duration of storage (TRECs: 46 ± 32, 65 ± 41, and 72 ± 53 copies/50 ng of genomic DNA, respectively; and KRECs: 71 ± 57, 67 ± 64, and 84 ± 65 copies/50 ng of genomic DNA, respectively). Similarly, there was no trend of decrease in means of TREC and KREC copies in relative quantification with increasing storage time.

To explore the relative role of T- and B-cell output in IH, the means of TREC and KREC copies were compared in patients with and without CaSRAbs or R110W mutation of *GCM2* gene and with respect to the presence or absence of the *HLA-A*26:01* allele. The data for these parameters were obtained from our previous studies [4, 7, 13]. The three nonoverlapping subsets of IH of CaSRAb and R110W mutation included patients: (i) with CaSRAb, but no R110W mutation of *GCM2* gene; (ii) with R110W mutation, but no CaSRAb; and (iii) who had neither CaSRAb nor R110W mutation. There was no significant difference in the mean of TRECs and KRECs in patients with and without CaSRAb and R110W mutation (Table 3). The median of absolute TRECs [per 50 ng of genomic DNA (IQR)] in patients with IH with the *HLA-A*26:01* allele (n = 60) and without this allele were comparable [45 (29 to 80) and 48 (35 to 93); $P = 0.27$]. Similarly, the medians of absolute KRECs were not significantly different in patients with or without HLA-A*26:01 [60 (35 to 97) and 48 (29 to 77); $P = 0.22$].

3. Discussion

Mechanisms of autoimmunity development in most endocrine disorders are only partially known and far from clear in IH. One of the major hurdles has been lack of availability of parathyroid tissue in IH, and, consequently, we have been adopting indirect measures to study markers of autoimmunity in them. To assess the possibility of impaired central tolerance in IH, this study was planned with several strengths, including a large group of patients with this disease, tools such as the TREC and KREC assays, patients with T1D and AT as autoimmune disease controls, and healthy subjects as general controls. The comparable age of all groups from the second to third decade of life precluded major confounding effects of

Table 3. Differences in the TREC and KREC Copies [Mean ± SD and Median (IQR)] in Subsets of Patients With IH With CaSRAb and *GCM2*-R110W Gene Mutations

Parameters	Subsets of IH: Subset a: With CaSRAb (>3.0 SDS) (n = 13)	Subsets of IH: Subset b: With *GCM2*-R110W Mutation (n = 15)	Subsets of IH: Subset c: Without CaSRAb or *GCM2*-R110W (n = 99)	Intersubset Differences (*P* Value): a vs b	a vs c	b vs c
TREC copies/50 ng of genomic DNA	73 ± 51 46 (37–111)	76 ± 67 48 (23–107)	62 ± 46 48 (34–77)	0.91	0.68	0.99
TREC copies /10^3 cells	30 ± 16 28 (20–40)	24 ± 16 25 (9–33)	28 ± 20 22 (13–33)	0.46	0.47	0.99
KREC copies/50 ng of genomic DNA	87 ± 114 48 (36–82)	60 ± 36 51 (36–66)	73 ± 58 54 (32–100)	0.99	0.99	0.99
KREC copies /10^3 cells	31 ± 23 23 (18–35)	21 ± 10 19 (13–28)	32 ± 23 24 (17–43)	0.40	0.99	0.22

altered T-cell output due to age-related thymic involution [32, 33]. The storage of DNA in IH had no limiting effect on the TREC and KREC assay, as revealed by the results of stratified analysis using samples of IH stored from 1998 to 2016. Though there were intergroup differences in the BMI, the normal mean BMI in the four study groups could have excluded its major effect on TREC and KREC molecules [34]. Similarly, despite differences in the sex ratio in the four study groups, TRECs and KRECs were comparable between males and females akin to the observation of Geenen *et al.* [32], but unlike the results of Pido-Lopez *et al.* [35].

In the current study, mean values of TRECs and KRECs in the IH group were similar to those of HC, but lower than those with T1D. Comparable TRECs and KRECs between IH and HC could indicate that central tolerance was not impaired in IH. However, enhanced peripheral proliferation of T and B cells can also result in a similar observation even in the presence of impaired central tolerance. Indeed, increased proliferation of T and B cells in the peripheral circulation would result in a dilution of TRECs and KRECs because these circular DNA molecules do not replicate with cell division [19]. This possibility seems unlikely because when a subset of patients with IH was reassessed after a decade of follow-up, there was no remarkable change in the detection of TRECs and KRECs.

In the IH subgroup analysis, TREC and KREC levels were also comparable in patients with and without CaSRAbs. Because our previous studies suggested notable association with *HLA-A*26:01* allele in IH [13], patients with IH were stratified based on presence or absence of *HLA-A*26:01* allele. However, there was no noteworthy difference in the TREC and KREC values between the two groups. Therefore, our data indicate that unlike in T1D and AT, there is a normal central immune tolerance in IH.

Impaired central tolerance in autoimmune endocrine disorders is a relatively new concept. However, up to this date, only a few studies have investigated circulating TRECs in patients with T1D [15], Hashimoto thyroiditis [36], and Graves disease [16, 37], and there are no data regarding KREC measurement in any autoimmune endocrine disorders. Hofer *et al.* [15] observed increased circulating TRECs in 38 patients with T1D with mean age of 10 to 15 years. Circulating TRECs correlated with duration of disease and insulin therapy. Though we had observed increased TRECs in our large cohort of T1D, the increase did not correlate with duration of diabetes. This can be due to the higher mean age (32 years) and large cohort size of our study. Circulating KRECs were at normal levels in T1D, indicating importance of T cells over B cells in the autoimmune process of T1D. In fact, pancreatic biopsy of patients with newly diagnosed T1D showed $CD8^+$ T lymphocytes as the predominant infiltrating cells, with B lymphocytes being fewest in number [38].

Similarly, as previously observed [36], we found reduced TRECs in AT, together with the highest values of KRECs. In contrast, patients with Graves disease showed an increased influx of thymic emigrants in the thyroidectomy specimens and peripheral blood [16, 37]. The relatively prevalent role of T cells in Graves disease and of B cells in AT is also supported by the immunostaining of lymphocyte subpopulations in thyroid tissues of patients with these diseases [39]. Indeed, a strong immunostaining of $CD20^+$ B cells was observed in 100% of the thyroidectomy specimens of 17 patients with Hashimoto thyroiditis, but in only 50% of 18 patients with Graves disease [39]. Similarly, a higher proportion of plasma $CD79^+$ cells, which help in pro–B-cell to pre–B-cell development, in comparison with T cytotoxic $CD8^+$ cells (32% vs 21%) was found in the thyroid specimens of 30 children with AT [40]. Although thyroid hormones are important for T- and B-cell development [16, 41] and although TRECs were found to be higher in a hyperthyroid state in Graves disease [16], in this study, values of TRECs and KRECs were comparable in AT with TSHs <10 and ≥ 10 IU/mL.

Although the present study indicates that central tolerance is not impaired in IH, it has some limitations. We had assessed TRECs and KRECs in the stored DNA samples prepared from total lymphocyte population, and, therefore, we do not have information regarding the numbers of these markers of thymic and bone marrow output in in $CD4^+$ or $CD8^+$ T cells. Our earlier studies suggested a possibility of a substantial role of $CD8^+$ T cells in IH, with considerable association with the *HLA-A*26:01* allele [13, 14]. Thus, assessment of TREC concentration in the CD8 T-cell subset might reveal differences between patients with IH and controls. In addition, recent thymic migrants can also be sorted out, taking advantage of the expression of CD45RA and CD31 markers in T cells [42]. The measurement of this T-cell subset could be useful as an alternative approach to confirm the observations of our study. Similarly, sorting of B lymphocytes, based on the expression of CD19 and CD10 molecules and the lack of CD27 marker, indicative of recent bone marrow migrants, could add further information on central tolerance of B cells in IH [43, 44].

In conclusion, the current study indicates that patients with IH have TREC and KREC values comparable to those of HCs. In contrast, patients with T1D exhibit a substantial increase in circulating TRECs, whereas patients with AT show a preferential increase of KRECs, suggesting an increased output of T and B cells from their respective production sites in these two diseases, respectively. These findings are reminiscent of our earlier observation, which indicated no major increase in coexistent thyroid autoimmunity in IH, unlike in patients with T1D, AT, and premature ovarian failure [3]. Further studies such as of the role of regulatory T cells involved in peripheral immune tolerance would be helpful to understand the autoimmunity-related loss of parathyroid tissue in IH.

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