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Autoimmune Predisposition in Down Syndrome May Result from a Partial Central Tolerance Failure due to Insufficient Intrathymic Expression of *AIRE* and Peripheral Antigens

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Down syndrome (DS), or trisomy of chromosome 21, is the most common genetic disorder associated with autoimmune diseases. Autoimmune regulator protein (AIRE), a transcription factor located on chromosome 21, plays a crucial role in autoimmunity by regulating promiscuous gene expression (pGE). To investigate if autoimmunity in DS is promoted by the reduction of pGE owing to dysregulation of *AIRE*, we assessed the expression of *AIRE* and of several peripheral tissue-restricted Ag genes by quantitative PCR in thymus samples from 19 DS subjects and 21 euploid controls. Strikingly, despite the 21 trisomy, *AIRE* expression was significantly reduced by 2-fold in DS thymuses compared with controls, which was also confirmed by fluorescent microscopy. Allele-specific quantification of intrathymic *AIRE* showed that despite its lower expression, the three copies are expressed. More importantly, decreased expression of *AIRE* was accompanied by a reduction of pGE because expression of tissue-restricted Ags, CHRNA1, GAD1, PLP1, KLK3, SAG, TG, and TSHR, was reduced. Of interest, thyroid dysfunction (10 cases of hypothyroidism and 1 of Graves disease) developed in 11 of 19 (57.9%) of the DS individuals and in none of the 21 controls. The thymuses of these DS individuals contained significantly lower levels of AIRE and thyroglobulin, to which tolerance is typically lost in autoimmune thyroiditis leading to hypothyroidism. Our findings provide strong evidence for the fundamental role of AIRE and pGE, namely, central tolerance, in the predisposition to autoimmunity of DS individuals. *The Journal of Immunology*, 2014, 193: 3872–3879.

Down syndrome (DS), also known as trisomy 21, is the most common genetic disorder of live-born infants and has been long considered a model of accelerated aging (1). Cardiac malformations and predisposition to Alzheimer disease are prominent features of this syndrome (2, 3). Various immunological abnormalities are also found among DS patients, including thymic atrophy, high frequencies of hematologic malignancies, susceptibility to infections, and, most remarkably, a high incidence of autoimmune diseases (4–6). In DS, autoimmunity may affect both endocrine (thyroid, pancreatic islets, and adrenal gland) and nonendocrine organs (stomach and small bowel), which results in a high incidence of thyroiditis and hypothyroidism, type 1 diabetes, Addison disease, and celiac disease. Other associated immune-mediated disorders include alopecia areata, chronic autoimmune hepatitis, and primary sclerosing cholangitis (6–11). Autoantibodies are frequently found in DS subjects, even when there is no apparent overt autoimmune disease.

Although it is widely accepted that autoimmunity requires a failure of immunological tolerance to self-antigens, how this originates in most cases remains unknown. However, solid evidence exists that genetic factors play a major role. Because the primary genetic anomaly in DS is well defined, analyzing autoimmune disease in DS can contribute to advancing our understanding of both autoimmunity and DS.

It is recognized that the spectrum of autoimmune diseases that DS subjects have is reminiscent of that seen in the rare autosomal recessive disease autoimmune poly-endocrinopathy-candidiasis-ectodermal dystrophy syndrome, also called autoimmune polyendocrine syndrome type 1. This disease results from inactivating mutations in the *AIRE* gene (12). This gene is selectively expressed in the thymic medulla and evidence is considerable that autoimmune regulator protein (AIRE) influences the transcription of many genes that encode for peripheral tissue-restricted Ags (TRAs) (12, 13). This activity has been designated "promiscuous gene expression" (pGE)—namely, expression in the thymus of a much broader spectrum of self-antigens than would be expected for a lymphoid organ. There is also evidence that this pGE pattern protects against organ-specific autoimmune disease, presumably by enhancing both negative selection and the generation of natural regulatory cells (14, 15).

The *AIRE* gene is located in the 21q22.3 region, and thus DS subjects carry three copies of this gene (16). Paradoxically, however, it was recently reported that the level of AIRE expression in the thymus of DS subjects is clearly reduced (17). This rather unexpected finding may help in understanding the predisposition to

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Abbreviations used in this article: AIRE, autoimmune regulator protein; ASTQ, allele-specific transcript quantification; CV, coefficient of variation; DS, Down syndrome; gDNA, genomic DNA; HVH, Vall d'Hebron University Hospital; KLK3, kallikrein-related peptidase 3; KRT14, cytokeratin 14; miRNA, microRNA; mTEC, medullary thymic epithelial cell; pGE, promiscuous gene expression; PLP1, proteolipid protein 1; SAG, retinal S-antigen; SNP, single nucleotide polymorphism; TG, thyroglobulin; TRA, tissue-restricted Ag; TSHR, thyroid-stimulating hormone receptor.

autoimmunity with DS. Because understanding AIRE expression regulation and its consequences is of considerable interest, we wanted to garner insights into the mechanisms that result in its reduced expression in the thymus of DS subjects and to test whether decreased AIRE expression might affect pGE in the thymus of these subjects.

Materials and Methods

Samples and patients

Thymus glands were obtained from archived samples collected since 1995: 19 DS subjects, all with proven trisomy 21, and 21 children without DS. All subjects underwent corrective heart surgery at the Division of Pediatric Surgery of Vall d'Hebron University Hospital (HVH), a hospital affiliated with the Faculty of Medicine of the Autonomous University of Barcelona (Table I). Children without DS but with congenital heart defects were otherwise healthy and served as controls. Informed consent was obtained from the parents or legal wards of the patients, and this study protocol was reviewed and approved by the HVH Clinical Research Ethics Committee. Thyroid status is based on the annotations in the paper and/or electronic clinical records of HVH and the primary care center through the Shared Clinical Records of Catalonia (www.ticsalut.cat) and is summarized in Table I.

RNA extraction and gene expression analysis

Genomic DNA (gDNA) and total RNA were isolated from total thymus using standard methods (Maxwell 16 System; Promega). An additional step of DNase I treatment was used for all RNA samples (DNA-*free* Kit; Ambion). RNA integrity was assessed by denaturing agarose gel, and 2 μg total RNA was retrotranscribed to cDNA using oligo-dT primers and a SuperScript III First-Strand Synthesis System. Relative gene expression was determined by quantitative real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocol and normalized to GAPDH mRNA. Reactions were run on a LightCycler 480 (Roche) in triplicate, and the average CT values were used for statistical analysis. The coefficient of variation (CV) was repeatedly <15%.

Allele-specific transcript quantification

Allele-specific transcript quantification (ASTQ) analysis was done as previously described (18, 19). On the basis of data from the dbSNP (http://www.ncbi.nlm.nih.gov/snp/) and HapMap databases (20), we selected single nucleotide polymorphism (SNP) rs1055311 (C/T) in exon 6 of the *AIRE* gene as a target. Total RNA and DNA were extracted from thymus samples of DS subjects. Allele-specific gene expression was determined by qPCR amplification with specific primers and FRET probes designed for this SNP. Detailed information on generation of standards and calibration curves is described elsewhere (18, 19). Primers and FRET probes used for AIRE rs1055311 SNP were as follows: primer sense, 5′-GCGGCTCCAAGAAGTGCAT-3′; primer antisense, 5′-GCTCCCTTGGCTCGAACC-3′; FRET probe sensor, 5′-AGGTTGGCGGGGAGTTCT^{Fluos}-3′; FRET probe anchor, 5′-^{640}CACTCCCAGCAAGTTCGAAGACTCCG^{Phosphate}-3′. Reactions were run on a LightCycler 480 (Roche). Each sample was run in triplicate, and tests were independently repeated twice. Reference standards were included during each run. The CV was repeatedly < 10%, which indicated reproducibility.

Immunofluorescence analysis

Frozen thymus sections (4 μm thick) were stained using an indirect immunofluorescence method, as previously described (21). Briefly, frozen cryosections were air dried, and nonspecific binding was reduced by incubation for 1 h with 1% (w/v) BSA in PBS. Sections were then incubated with primary Abs for 1 h, extensively rinsed in PBS, incubated with a secondary Ab for 30 min, and rinsed again with PBS; all steps were done at room temperature. The primary Abs used were as follows: mouse monoclonal anti-cytokeratin 14 (KRT14) (clone NCL-LLO02; Novacastra), goat polyclonal anti-AIRE1 (sc-17986; Santa Cruz Biotechnology), and rabbit polyclonal anti-CD20 (PA-1671; ThermoFisher) used at 1:100 dilution. Secondary Abs, anti-mouse or anti-rabbit Alexa 488 and anti-goat Alexa 546, were purchased from Molecular Probes and used at 1:500 dilution. Standard controls included the secondary Ab without using the primary or isotype control. Slides were mounted with SlowFade Gold (Molecular Probes) and examined under an Olympus BX61 microscope. Images were acquired using an Olympus DP72 camera and were analyzed using National Institutes of Health Image J software (22).

Statistical analysis

The R statistical software package (23) (www.R-project.org) was used for statistical analysis and data plotting. Because gene expression values were not normally distributed, a Wilcoxon signed rank test was used for statistical comparisons. Associations between the expressions of pairs of genes were assessed using nonparametric Spearman rank correlation tests. A p value < 0.05 was considered significant.

Results

High prevalence of thyroid dysfunction among DS subjects

Table I summarizes the main features of the samples included in this study. Thymus glands were obtained from 19 DS subjects, all with proven trisomy 21, and 21 children without DS. The DS and control groups were comparable, as there were no significant differences in age (2.03 y; range 4 mo–10 y versus 1.46 y; range 5 d–6 y, respectively; $p = 0.27$) or gender (9 females and 10 males versus 8 females and 13 males, respectively; $p = 0.55$). DS subjects and healthy controls were followed up over a period of 21 and 17 y, respectively. One of the patients had congenital hypothyroidism, as assessed by the neonatal screening program, and it has not been included in the statistical analysis of the last figure, as it is not autoimmune. Thyroid dysfunction of presumably autoimmune origin was detected in 11 of 19 cases (57.9%) of which 6 (31.6%) had hypothyroidism and 4 (21%) were subclinical and one suffered Graves disease. One of the hypothyroid patients also had celiac disease. Overall, this was a >50-fold increase compared with the prevalence of hypothyroidism in the general population and was in line with the previously reported higher prevalence of hypothyroidism and earlier age at onset observed for this genetic condition (9, 24). On the whole, this was consistent with hypothyroidism being the commonest form of thyroid disorder associated with DS (24).

AIRE *gene expression is reduced in the thymus of DS individuals*

AIRE expression in thymic medullary epithelial cells (mTECs) is critical for central tolerance to self. To investigate *AIRE* expression in our samples, we determined *AIRE* mRNA levels by quantitative real-time PCR using TaqMan probes for cDNA samples obtained from total thymus tissues of 19 DS subjects and 21 controls. We normalized the results to those of GAPDH mRNA, which is not influenced by *AIRE* gene expression (14, 25). As shown in Fig. 1A, *AIRE* expression was significantly reduced in the thymus tissues of DS subjects compared with the control group (222.9 ± 138.5 versus 466.9 ± 292.1; $p = 0.0003$). This decrease in *AIRE* mRNA levels represented a reduction of 47.7% in DS subjects as compared with controls. Boxplots also showed that the variability of *AIRE* expression levels was similar in both groups (CVs of 62% and 63%, respectively).

In the thymus, because *AIRE* is primarily expressed in the thymic epithelial compartment (mTECs) (12), we also determined *KRT14* expression, a well-established marker of mTECs (26), and assessed possible correlations between *KRT14* and *AIRE* expression. No difference was observed in *KRT14* levels between DS subjects and healthy controls ($p = 0.63$, Fig. 1B). This finding indicated that the reduced *AIRE* expression was not due to a reduced medullary compartment. As expected, the *KRT14* and *AIRE* expression levels were significantly positively correlated ($r = 0.55$; $p = 0.0004$; Fig. 1C) and were similar in DS and control subjects. In fact, *AIRE* expression is also significantly reduced in DS patients compared with the control group when normalized to *KRT14* mRNA levels ($p = 0.006$; Fig. 1D). These findings confirmed that the observed reduction in AIRE expression was specific for DS subjects, as previously reported (17).

Table I. Samples included in the study

Healthy Donors			DS Patients				
Code	Gender	Age[a]	Code	Gender	Age[a]	Thyroid Dysfunction	Follow-up Period (y)
H1	Male	2 y	DS1	Male	6 y	No	26
H2	Female	8 d	DS2	Female	10 y	Graves disease	25
H3	Male	6 y	DS3	Male	6 y	Hypothyroidism	24
H4	Male	11 mo	DS4	Male	4 mo	NA	NA
H5	Male	1 y	DS5	Female	9 mo	No	17
H6	Male	7 d	DS6	Female	5 mo	Hypothyroidism[b]	16
H7	Male	1 mo	DS7	Male	11 mo	Hypothyroidism[b]	16
H8	Female	3 y	DS8	Female	2 mo	No	15
H9	Male	12 d	DS9	Male	7 mo	No	15
H10	Female	5 y	DS10	Female	6 mo	Subclinical hypothyroidism	11
H11	Male	3 mo	DS11	Female	8 y	No	22
H12	Male	1 mo	DS12	Female	7 mo	Subclinical hypothyroidism	9
H13	Male	11 d	DS13	Female	8 mo	Subclinical hypothyroidism	7
H14	Female	1 y	DS14	Male	1 y	Subclinical hypothyroidism	8
H15	Female	3 mo	DS15	Male	11 mo	Hypothyroidism, CD	8
H16	Male	3 mo	DS16	Male	5 mo	Congenital hypothyroidism	7
H17	Female	3 y	DS17	Female	8 mo	No	7
H18	Female	7 mo	DS18	Male	4 mo	Hypothyroidism	7
H19	Male	5 d	DS19	Male	5 mo	Hypothyroidism	5
H20	Male	3 y					
H21	Female	4 y					

[a]Age at thymectomy.
[b]Exitus.
CD, celiac disease.

AIRE-positive cells are reduced in the thymic medulla of those with DS

We next examined the tissue expression and distribution of AIRE expression using double-immunofluorescence staining for the tissue sections of five DS subjects and five healthy controls. Thymus tissue sections were stained for KRT14 and AIRE. The medulla was identified based on its high KRT14 content and the presence of Hassall bodies. As shown in Fig. 2, AIRE-positive cells showed typical nuclear body–like staining patterns and were observed in the thymic medullary epithelial compartment, as identified by double staining with KRT14. Significantly fewer AIRE-positive cells were found in the thymic medulla of DS patients compared with those in the control group (37 ± 23 versus 116 ± 56 cells per square millimeter, respectively; $p < 0.0001$). DS subjects exhibited a poorly defined thymic compartment compared with the control group, as determined by KRT14 staining. We performed morphometric analysis in a subset of five DS individuals and healthy controls, matched for age, and found no statistically significant differences in the percentage of cortex (32.7 ± 15.7 versus 42.6 ± 5.2) or medulla (67.3 ± 25.7 versus 57.4 ± 5.2) between DS and controls. However, we did observe a statistically significant difference in the number of Hassall bodies per square millimeter of tissue (6.15 ± 1.06 versus 24.6 ± 4.5, respectively; $p < 0.0001$) between these two groups of patients. Similar results were obtained with double staining of thymic sections with AIRE and HLA-DR (data not shown). We also explored the presence of lymphoid follicles with germinal centers, using an anti-CD20–specific Ab. Despite finding a good number of B cells in both groups of patients, this method did not reveal recognizable primary or secondary lymphoid follicles (Fig. 2E, 2F).

Tissue-restricted Ag expression is reduced in the thymus of those with DS

Intrathymic expression of TRAs resulting from pGE is thought to be a key element for inducing central tolerance, and AIRE has been demonstrated to have a central role in this process. We hypothesized that the observed marked decrease in AIRE expression in DS patients would result in a reduced expression of those TRAs that were AIRE dependent (27). To confirm this, we evaluated seven TRA genes with varying AIRE dependencies by quantitative PCR. TRAs analyzed were the following: *CHRNA1* (cholinergic receptor, nicotinic, α 1), primarily expressed in skeletal muscle; *GAD1* (glutamate decarboxylase 1, 67 KDa), primarily expressed in brain and pancreatic islets; proteolipid protein 1 (*PLP1*), the major myelin protein in the CNS; kallikrein-related peptidase-3 (*KLK3*), which encodes for prostate-specific Ag; retinal S-antigen (*SAG*), expressed in the retina; thyroglobulin (*TG*) expressed in the thyroid; and thyroid-stimulating hormone receptor (*TSHR*), also primarily expressed in the thyroid.

Fig. 3 shows that all of these TRAs had reduced expression in the thymus of DS subjects compared with controls, although only the differences in *KLK3* (4.64 ± 2.44 versus 10.16 ± 6.59; $p = 0.0019$) and *SAG* (5.39 ± 2.57 versus 9.41 ± 6.13; $p = 0.0004$) expression were statistically significant. The two thyroid autoantigens analyzed, *TG* and *TSHR,* were at the limit of significance ($p = 0.088$ and 0.065, respectively), probably because of the small number of samples analyzed and the intrinsic high interindividual variability of gene expression.

To further explore the influence of AIRE on TRA expression, we assessed the correlations between the expressions of *AIRE* and each TRA. Fig. 4 shows that all but one TRA (*PLP1*, $r = 0.37$; $p = 0.1641$) were significantly positively correlated with *AIRE* expression. Of interest, the correlation coefficients between *AIRE* and TRAs were similar in the DS and control groups, which suggested that the role of AIRE in controlling the promiscuous expression program did not differ between DS subjects and controls. Of note, the two TRAs that showed the highest correlations with *AIRE* (*KLK3,* $r = 0.79$ and *SAG*, $r = 0.76$) also had the greatest significant reduction in the thymus of DS subjects (Figs. 3, 4). This finding suggested that AIRE-dependent TRAs were more likely to be sensitive to moderate reductions in AIRE expression.

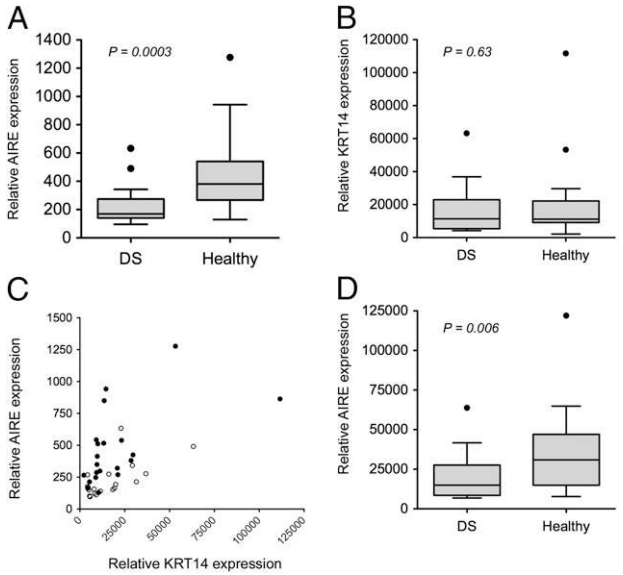


FIGURE 1. Reduced *AIRE* gene expression in thymus from children with DS. Quantitative PCR was performed in total thymus samples of DS ($n = 19$) and control ($n = 21$) children. Boxplots represent the quartiles and range of relative mRNA expression values of (**A**) AIRE mRNA relative expression normalized to GAPDH mRNA, (**B**) KRT14 mRNA relative expression normalized to GAPDH. Each sample was tested in triplicate, and the SD was always <10% of the mean value. (**C**) The values of relative KRT14 and AIRE expression in total thymus for each sample are shown (●, healthy individuals; ○, DS children). No differences were observed between DS and control children. Each sample was tested in triplicate, and the SD was always <10% of the mean value. Significantly positive correlation of KRT14 and AIRE expression, Spearman $r = 0.55$; $p = 0.0004$. The p value is two-tailed. (**D**) Boxplots showing significantly reduced relative expression of AIRE mRNA values in DS individuals compared with healthy patients normalized to KRT14 mRNA (the mean and SD in DS and control patients are as follows: 19603 ± 14727 versus 36393 ± 26056). Each sample was tested in triplicate and the SD was always <10% of the mean value. For all graphs, further outliers are depicted as ●.

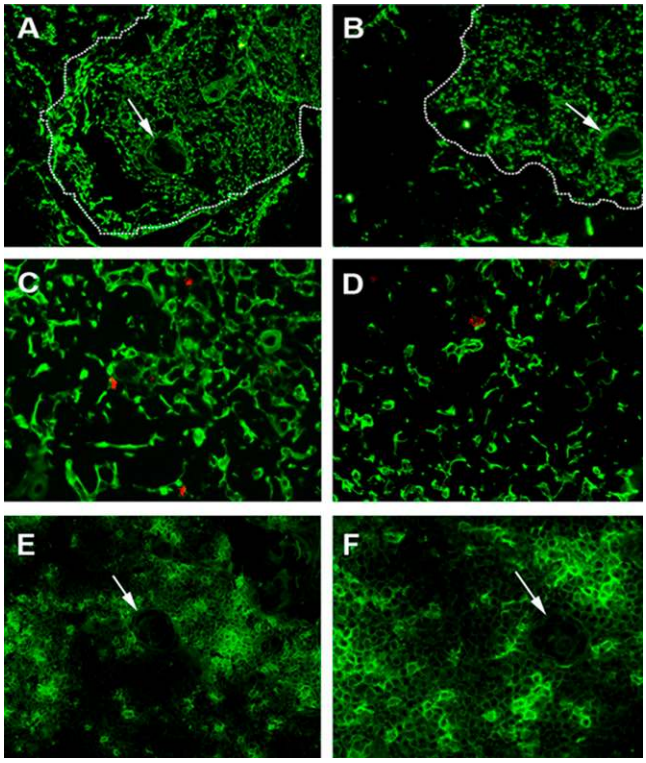


FIGURE 2. Reduced AIRE$^+$ cells in thymus from children with DS. Immunofluorescence of representative frozen thymic sections from control (*left panels*, **A**, **C**, and **E**) and DS (*right panels*, **B**, **D**, and **F**) patients were single stained with anti-KRT14–specific Ab as a thymic medullary marker [(A) and (B), original magnification ×100, KRT14 shown in green] or stained in combination with anti-AIRE–specific Ab [(C) and (D), original magnification ×400, KRT14 shown in green and AIRE shown in red]. (E) and (F) original magnification ×20 and ×40 objective micrographs, respectively, of DS frozen thymic sections stained for B lymphocytes using anti-CD20. Cell aggregates are clearly visible, but they do not adopt lymphoid follicle with germinal center architecture. The area and number of aggregates were not different between normal and DS samples. Broken lines indicate the corticomedullary borders, and arrows indicate Hassall corpuscles.

Three copies of the AIRE *gene are expressed in the thymus of those with DS*

Despite carrying an extra copy of chromosome 21, on which the *AIRE* gene is located, *AIRE* expression in those with DS was reduced 2-fold. To assess the cause of this discrepancy, we investigated the contributions of the three copies of the *AIRE* gene to total *AIRE* expression in the thymus of those with DS. To this end, we compared the relative allelic expressions of *AIRE* transcripts by ASTQ analysis, using a known SNP, rs1055311, located within exon 6 of the *AIRE* gene as a marker (see *Materials and Methods* and Fig. 5A). We calculated the C/T ratios for this SNP in euploid thymic samples and subsequently compared these samples with those of DS subjects (Fig, 5B).

The genomic ratios of C/T alleles for the healthy controls ranged from 1.41 to 1.48 for the healthy samples that were used as reference values (differential binding of the sensor FRET probe to each allele explains why the calculated ratios were above 1) (see Fig. 5B). We found that 31.6% of DS subjects (6 of 19) were heterozygous at this SNP site, which was consistent with data reported in the dbDNP and HapMap databases. Five DS patients had a C/C/T genotype, and one had a C/T/T genotype, which agreed with the minor allele frequency of T reported for whites (minor allele frequency = 0.26) (20). As shown in Fig. 5B, C/T allele ratios of gDNA samples of DS subjects were concordant with their allele dose (from 2.80 to 2.86 for the C/C/T samples and 0.71 for the C/T/T sample). Then, we calculated the relative expression of the rs1055311 alleles in the thymic cDNA of heterozygous DS individuals. In all cases, the ratio was concordant with their allelic dosage. This finding established that the three copies of the *AIRE* gene were expressed in the thymus of those with DS.

Intrathymic AIRE *and TG expression is reduced in those with DS and hypothyroidism*

TSHR, TPO, and TG have been identified as thyroid targets in thyroid autoimmune disease (28). In our study, TPO could not be amplified, and TSHR and TG expression was decreased in the thymus of DS individuals, but the difference did not reach significance compared with the control group. However, when we looked specifically at the levels of TG in the thymus of the individuals who have developed hypothyroidism in the course of this 20-y study, the reduction was clearly significant when compared with healthy controls (76.91 ± 19.93 versus 114.8 ± 43.45; $p = 0.0029$) (Fig. 6A). Furthermore, the reduction in intrathymic *AIRE* expression is greater in DS individuals with hypothyroidism than in those without hypothyroidism, compared with controls (187.5 ± 82.09 and 291.5 ± 196.5 versus 466.9 ± 292.1; $p = 0.0009$ and p = NS, respectively) (Fig. 6B). However, the levels of intrathymic expression of a thyroid autoantigen

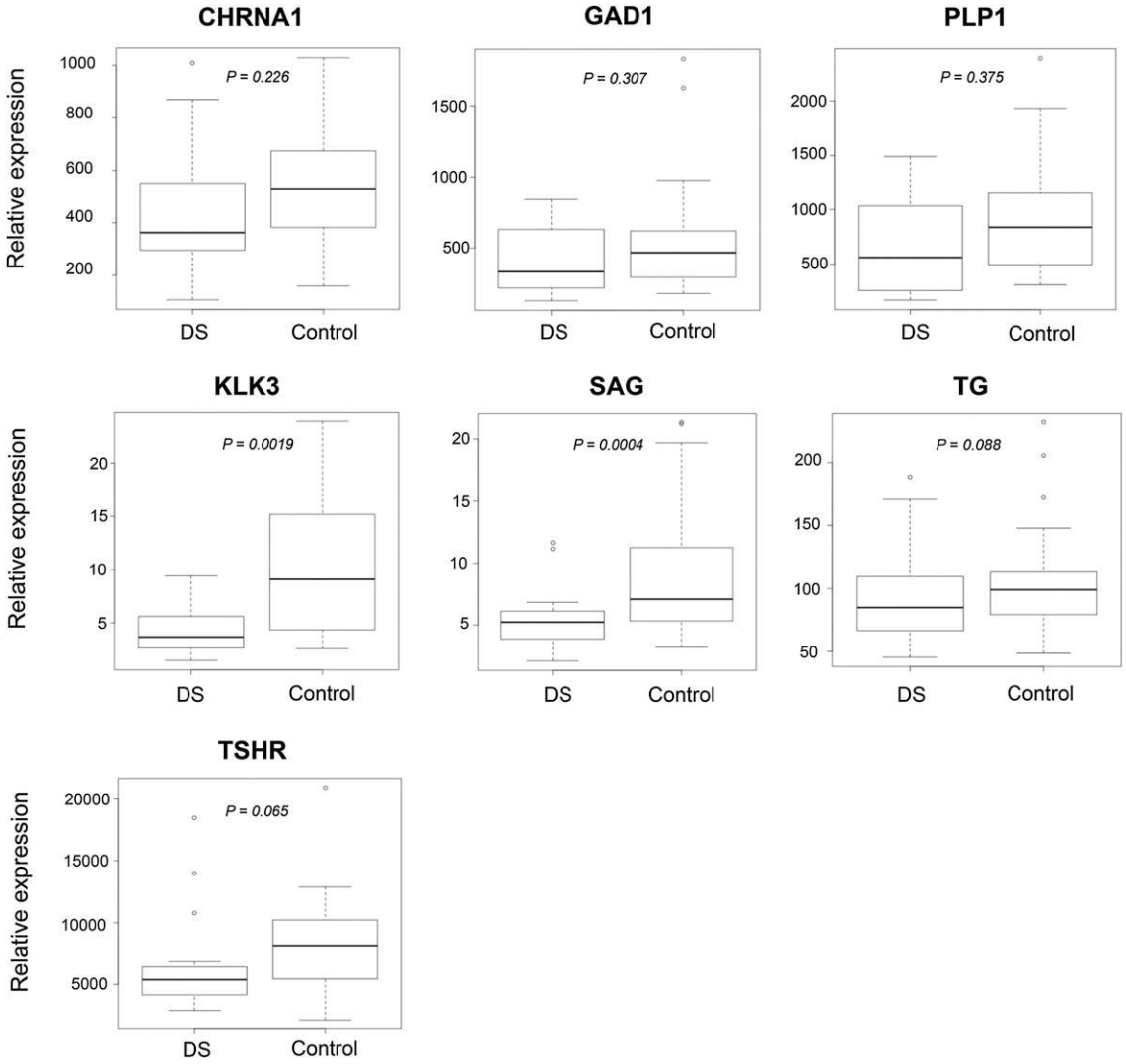


FIGURE 3. Reduced TRA gene expression in thymus from children with DS. Relative expression of *CHRNA1, GAD1, PLP1, KLK3, SAG, TG,* and *TSHR* was measured in total thymus of DS ($n = 19$) and control ($n = 21$) children. Each sample was tested in triplicate and the SD was always <10% of the mean value. Results are plotted using box-and-whisker graphs. Any further outliers are represented as ●. The median of each box is represented with a thicker line. The mean and SD for each TRA in DS and control patients are as follows: *CHRNA1* (454.3 ± 247.0 versus 549.4 ± 240.4), *GAD1* (416.4 ± 238.3 versus 576.4 ± 433.4), *PLP1* (685.2 ± 458.6 versus 888.5 ± 545.2), *KLK3* (4.635 ± 2.442 versus 10.16 ± 6.587), *SAG* (5.394 ± 2.565 versus 9.745 ± 5.887), *TG* (92.39 ± 37.57 versus 113.1 ± 44.14), *TSHR* (6419 ± 3941 versus 8168 ± 4152), respectively.

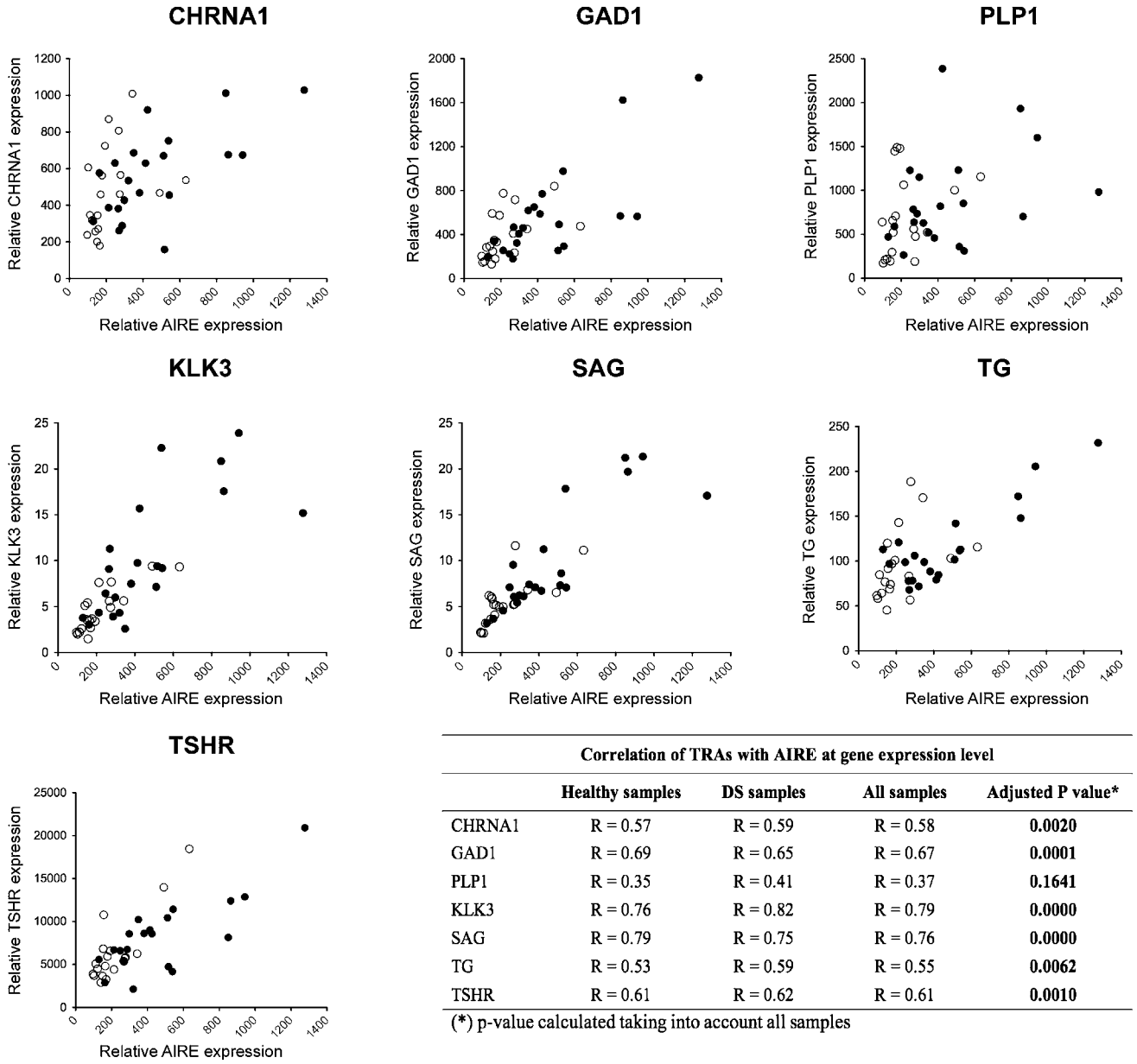
associated with Graves disease, *TSHR*, were similar between hypothyroid DS and healthy controls (Fig. 6C).

Discussion

In this study, we confirmed that there was a significant, specific reduction in *AIRE* expression in the thymus of those with DS (17) and that this resulted in clearly reduced pGE in these subjects. It is well established that, in the thymus, an adequate level of self-antigen expression is crucial for central tolerance, and that relatively modest reductions in the already low level of TRA expression may have a profound effect on the predisposition to autoimmunity (29). The critical role of TRA expression levels in mTECs is exquisitely illustrated in three human autoimmune diseases: type 1 diabetes mellitus (30, 31), myasthenia gravis (32), and Graves disease (19). For these diseases, genetic polymorphisms result in reduced mean expression levels of the target autoantigens (insulin in type 1 diabetes mellitus, α-chain of the acetylcholine receptor in myasthenia gravis, and thyrotropin receptor in Graves disease) in mTECs by a factor of 2 to 4; this is sufficient to confer a higher risk for the particular disease.

In the current study, we found that *AIRE* expression was 2-fold lower in the thymuses of those with DS and that this was accompanied by specific 2-fold reductions in *KLK3* and *SAG* expressions in those with DS. Of interest, these genes also showed the strongest positive correlations with *AIRE* expression, which is consistent with the proposition that the regulation of their expression is strongly influenced by *AIRE* and, thus, is affected by reduced *AIRE* expression. In contrast, PLP1 was not significantly correlated with AIRE expression or differences in the expression levels between DS subjects and controls. This finding is consistent with the reported AIRE-independent regulation of PLP1 expression (27). Overall, our results are in agreement with the range of expression levels assumed to be associated with an increased autoimmune susceptibility.

The reduced *AIRE* expression in those with DS in our study was not caused by quantitative changes in the medullary compartment, as assessed by *KRT14* mRNA expression. The decreased *AIRE* expression was not only accompanied by fewer AIRE-positive cells but also by changes in the organization of the medullary compartment in those with DS, as observed in our immunofluorescence



Correlation of TRAs with AIRE at gene expression level

	Healthy samples	DS samples	All samples	Adjusted P value*
CHRNA1	R = 0.57	R = 0.59	R = 0.58	**0.0020**
GAD1	R = 0.69	R = 0.65	R = 0.67	**0.0001**
PLP1	R = 0.35	R = 0.41	R = 0.37	**0.1641**
KLK3	R = 0.76	R = 0.82	R = 0.79	**0.0000**
SAG	R = 0.79	R = 0.75	R = 0.76	**0.0000**
TG	R = 0.53	R = 0.59	R = 0.55	**0.0062**
TSHR	R = 0.61	R = 0.62	R = 0.61	**0.0010**

(*) p-value calculated taking into account all samples

FIGURE 4. Correlation graphs of selected TRAs with *AIRE* expression levels. All but one TRA (PLP1) show a significant positive correlation with AIRE mRNA expression that is maintained in DS individuals. TRAs showing higher correlations with AIRE (KLK3 and SAG) are also the most significantly reduced in DS patients (see text for details). The ● correspond to healthy individuals and ○ to DS patients. The accompanying table summarizes the Spearman correlation values obtained individually for DS or healthy patients or simultaneously taking into account both groups.

analyses and in line with previous reports (5, 33). A lower number of Hassall corpuscles, as we observed, are associated with fewer terminally differentiated mTECs (34). The morphologic alterations in the medullary compartment observed in DS subjects were compatible with those observed in *AIRE*-deficient mice (35, 36), although the phenotype of DS subjects is obviously milder than that observed for these animals. These findings suggest that *AIRE* activity extends beyond its previously assigned role as a regulator of TRA expression to a role consistent with regulating thymic epithelium differentiation (37). These results argue for an inborn thymic defect in central tolerance induction, rather than precocious aging of the thymus, as previously hypothesized (38–41), and will be in agreement with recent studies on the function of the thymus in DS patients (17, 42).

One of the most intriguing aspects of AIRE in DS individuals is its counterintuitively reduced expression in those who carry an extra copy of chromosome 21, on which the *AIRE* gene is located. Accordingly, in these subjects, the *AIRE* gene should be overexpressed by 1.5-fold relative to the euploid state. Instead, *AIRE* expression in DS individuals was underexpressed by ~0.5-fold relative to healthy controls. This observation is uncommon, but not unique, to *AIRE* expression in those with DS. Two previous studies (43, 44) analyzed gene expression variations for 136 genes located on chromosome 21 in lymphoblastoid cell lines derived from DS subjects and found that only 30% of all trisomic genes had increased expression of ≥1.5-fold, whereas 55% exhibited compensated expression and 15% were overcompensated (43, 44).

In the current study, we determined the contributions of each of the three alleles of the *AIRE* gene in mRNA expression using an ASTQ technique. Our results showed that three copies of the *AIRE* gene were expressed in the thymuses from those with DS, thus ruling out chromosome inactivation or gene imprinting and

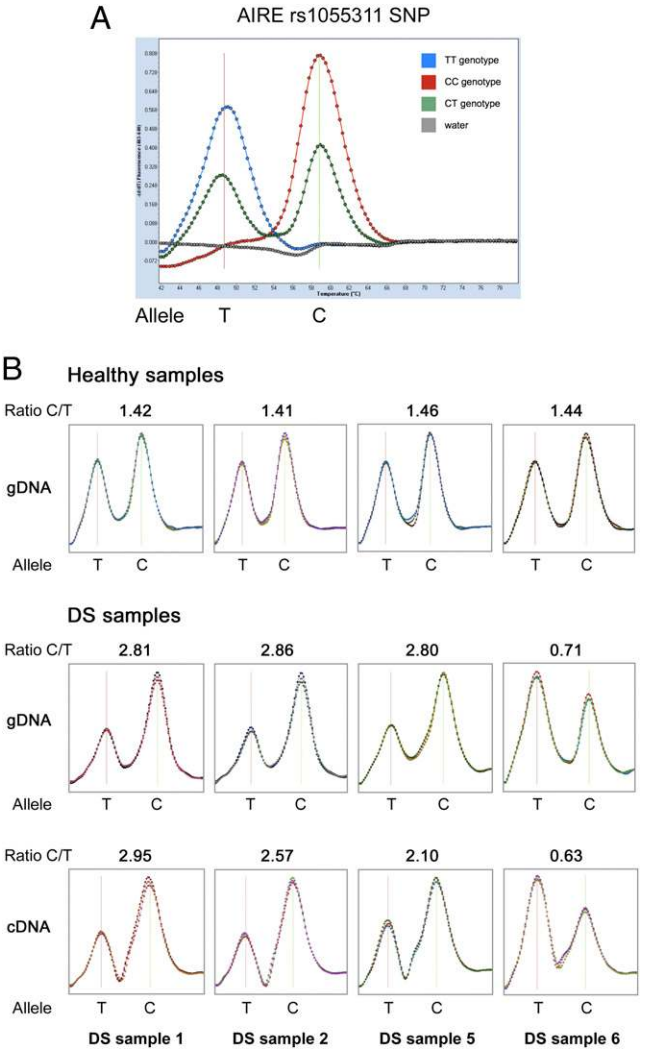


FIGURE 5. Expression of three copies of the *AIRE* gene in the thymus of DS children. (**A**) Melting curves produced by FRET probes for each rs1055311 genotype. Characteristic melting curves distinguish DNAs differing at only a single base pair. DNA melting is assayed by loss of fluorescence and, with the chart that plots the first negative derivative of the sample fluorescent curves, the melting temperature of each sample appears as a peak. (**B**) Melting curves reflecting the relative contribution of each of the two alleles. The ratio between the area's peaks, indicating the contribution of each allele in the sample, is shown. The gDNA of four heterozygous control individuals has been used as a 1:1 mixture reference. Using this 1:1 control mixture, we can readily correct for biases in FRET probe hybridization. Note that the gDNA profiles of all control samples are consistently the same. gDNA and cDNA melting profiles from four DS individuals with different heterozygous genotypes (three of them C/C/T and one of them C/T/T) are also shown.

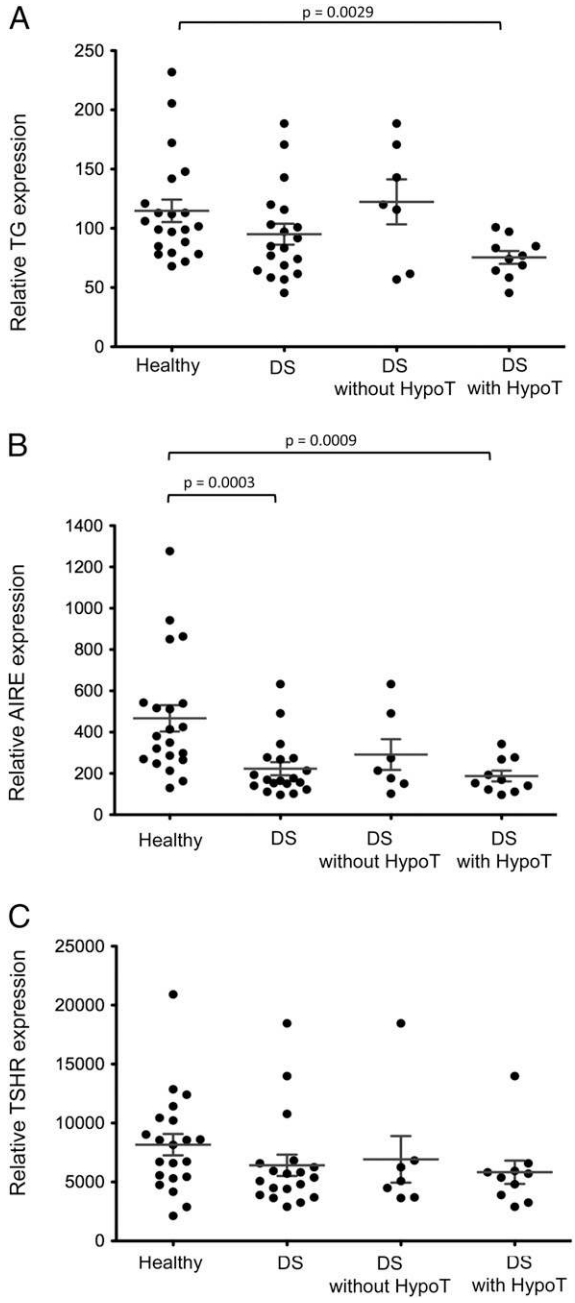


FIGURE 6. *AIRE* and *TG* expression is significantly reduced in the thymus of DS children with hypothyroidism. Relative TG (**A**), AIRE (**B**), and TSHR (**C**) mRNA expression was measured in the total thymus of control (n = 21) and DS (n = 19) children. DS individuals have been divided into those with hypothyroidism (with HypoT) and those without hypothyroidism (without HypoT). The patient with congenital hypothyroidism is not included in this analysis. Each sample was tested in triplicate, and the SD was always $<$ 10% of the mean value.

strongly suggesting that AIRE expression might be subject to epigenetic or other forms of posttranscriptional regulation that overcompensate for the excess of gene dosage. Because *AIRE* mRNA expression was significantly reduced in DS subjects, an attractive possibility is that *AIRE* expression is regulated by microRNAs (miRNAs). Indeed, chromosome 21 codes for at least seven miRNAs: miR-99a, let-7c, miR-125b-2, miR-155 and miR-802, and miR-nov1 and miR-nov2 (45). Although the targets and pathways of chromosome 21 miRNAs have not been determined, it has been proposed that overexpression of these miRNAs may contribute to the observed DS phenotypes, including increased incidence of autoimmune diseases (46–49). Of note, it has been recently reported that the regulation of pGE is under the control of miRNAs. However, the contribution of chromosome 21–encoded miRNAs is still under evaluation (50).

Illustrative of the high risk of autoimmunity in DS was the finding that hypothyroidism, presumably of autoimmune origin, had developed in more than half of the donors of the thymic glands analyzed and with significantly lower levels of AIRE and TG, an Ag to which tolerance is typically lost in autoimmune thyroiditis. Furthermore, one patient developed Graves disease and one hypothyroid patient had associated celiac disease. Thus, our findings provide evidence for the fundamental role of AIRE and pGE in preventing the development of autoimmunity in DS individuals and support the concept that

their predisposition to autoimmune disease is not due to premature immune senescence but to a qualitative failure of the thymus in the establishment of central tolerance. Furthermore, these results with DS individuals demonstrate how relatively minor reductions in pGE can have a major impact on predisposition to autoimmunity and support the concept that thymic central tolerance is a crucial checkpoint in the development of autoimmunity in general.

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Disclosures

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