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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J Immunol 2014; 193:3872-3879; Prepublished online 12 September 2014;
doi: 10.4049/jimmunol.1400223
http://www.jimmunol.org/content/193/8/3872

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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

Mireia Giménez-Barcons,* Anna Casteràs,‡ Maria del Pilar Armentegol,§ Eduard Porta,‡ Paula A. Correa,‡ Ana Marín,* Ricardo Pujol-Borrell,*§ and Roger Colobran*§

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.
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 theShared 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 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′-GTCCCTTGTCGTCACAGCC-3′; primer antisense, 5′-GTCCCTTGTCGTCACAGCC-3′; FRET probe sensor, 5′-AGTGGGCGGAGGTCTTCATGCGCG-3′; FRET probe anchor, 5′-AGTGGGCGGAGGTCTTCATGCGCG-3′; and FRET probe, 5′-AGTGGGCGGAGGTCTTCATGCGCG-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; Novocastra), 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).
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.

<table>
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<th>Code</th>
<th>Gender</th>
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Table I. Samples included in the study

- AIRE-positive cells
- DS: Down Syndrome
- CD: celiac disease
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 ●.

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...
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
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 \( \sim 0.5 \)-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 \( \sim 1.5 \)-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

\[ (*) \text{ p-value calculated taking into account all samples} \]
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 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.
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.

Acknowledgments

We thank Dr. Raül Abella and the team from the Division of Pediatric Surgery of Hospital Universitari Vall d’Hebron for providing thymus samples.

Disclosures

The authors have no financial conflicts of interest.

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