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THE JOURNAL of IMMUNOLOGY

This information is current as of September 13, 2011

Supplementary Data	http://www.jimmunol.org/content/suppl/2011/08/19/jimmunol.1003053.DC1.html
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Decreased AIRE Expression and Global Thymic Hypofunction in Down Syndrome

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The Down syndrome (DS) immune phenotype is characterized by thymus hypotrophy, higher propensity to organ-specific autoimmune disorders, and higher susceptibility to infections, among other features. Considering that *AIRE* (autoimmune regulator) is located on 21q22.3, we analyzed protein and gene expression in surgically removed thymuses from 14 DS patients with congenital heart defects, who were compared with 42 age-matched controls with heart anomaly as an isolated malformation. Immunohistochemistry revealed 70.48 ± 49.59 AIRE-positive cells/mm^2 in DS versus 154.70 ± 61.16 AIRE-positive cells/mm^2 in controls ($p < 0.0001$), and quantitative PCR as well as DNA microarray data confirmed those results. The number of FOXP3-positive cells/mm^2 was equivalent in both groups. Thymus transcriptome analysis showed 407 genes significantly hypoexpressed in DS, most of which were related, according to network transcriptional analysis (FunNet), to cell division and to immunity. Immune response-related genes included those involved in 1) Ag processing and presentation (*HLA-DQB1, HLA-DRB3, CD1A, CD1B, CD1C, ERAP*) and 2) thymic T cell differentiation (*IL2RG, RAG2, CD3D, CD3E, PRDX2, CDK6*) and selection (*SH2D1A, CD74*). It is noteworthy that relevant AIRE-partner genes, such as *TOP2A, LAMNB1,* and *NUP93,* were found hypoexpressed in DNA microarrays and quantitative real-time PCR analyses. These findings on global thymic hypofunction in DS revealed molecular mechanisms underlying DS immune phenotype and strongly suggest that DS immune abnormalities are present since early development, rather than being a consequence of precocious aging, as widely hypothesized. Thus, DS should be considered as a non-monogenic primary immunodeficiency. *The Journal of Immunology*, 2011, 187: 3422–3430.

D own syndrome (DS) represents the most common chromosomal disorder (∼1 in 700 live births) and results from total or partial trisomy of chromosome 21 (1–3). DS is associated with several complex clinical features including immunological abnormalities (4–8), and it has long been noticed that patients present abnormal thymuses, characterized by lymphocyte depletion, cortical atrophy, and loss of corticomedullary demarcation (9, 10). In addition to higher susceptibility to infections—as evidenced during the H1N1 2009 pandemic where the likelihood of death was 300 times greater for DS patients (11)—

DS is characterized by increased frequency of organ-specific autoimmune disorders and of lymphoid and myeloid leukemias, contrasting with a decreased risk for allergic diseases, particularly asthma (12, 13). Compared with the general population, the incidence of celiac disease, insulin-dependent diabetes mellitus, and hypothyroidism is, respectively 10–40, 6, and 4 times higher in DS patients, but Addison's disease, pernicious anemia, alopecia areata, vitiligo, and chronic hepatitis have also been reported (12, 14–16). This range of autoimmune disorders is reminiscent of autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), as are autoantibody reactivity patterns (17), and there is increased susceptibility to oral candidiasis (18) in APECED and DS. APECED is a monogenic disorder caused by loss-of-function mutations in *AIRE* (autoimmune regulator) (19, 20), which is located on 21q22.3.

In the search for the molecular basis for clinical features in DS patients, we have studied *AIRE* expression in DS thymus compared with that in age-matched individuals with heart defects as an isolated congenital malformation. We also studied global gene expression and transcriptional networks in thymuses of DS patients and controls, and we now report the finding that AIRE expression in DS thymic medullary epithelial cells is significantly reduced, data that were confirmed by quantitative PCR (qPCR). Additionally, the current analysis showed hypoexpression of genes related to immune response and to cell proliferation in DS thymus.

Materials and Methods

Specimens

Thymic tissues (corticomedullary sections) were collected between 2007 and 2010 from 14 DS (all with simple trisomy 21) infants and children (4 mo to 12 y old), all with congenital heart defects. These DS patients are being followed up: one died a few weeks after the surgery because of infectious

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complications; another developed Hashimoto thyroiditis at 3 y old. Control samples (42 thymuses) were from age-matched individuals with heart anomalies but no clinical signs of any other congenital malformation. All samples were collected during corrective cardiac surgery at the Hospital do Coração–Associação do Sanatório Sírio (São Paulo, Brazil). No tissue was removed for research purposes only. This study was approved by the hospital's ethics committee, and informed consent from the parents of all participating individuals was obtained.

Immunohistochemistry

Small pieces of each thymus were fixed in 10% neutral buffered formalin and processed into paraffin blocks. Thymus sections (4 μm) were deparaffinized and rehydrated through graded alcohols to water. For antigenic retrieval, sections were microwave-treated at 800 W in 10 mM Tris–EDTA buffer at pH 8 for 15 min and allowed to cool for 15 min. After washed in running tap water for 5 min, tissue endogenous peroxidase was inhibited with a solution of 3% H_2O_2 for 10 min at room temperature. Unspecific binding sites were blocked by incubation with 5% BSA (Sigma) diluted in 10 mM PBS pH 7.4 for 10 min. Slides were incubated overnight at 4°C with a polyclonal rabbit anti-AIRE serum (sc-33188; Santa Cruz Biotechnology, Santa Cruz, CA) and with an affinity purified rat anti-human anti-FOXP3 14-4776-82 (eBioscience, San Diego, CA). Primary Ab was blown off, and the slides were then incubated with the Universal LSAB+ Kit/HRP (DakoCytomation, Carpinteria, CA). The reaction was developed with the substrate in 3,3′ diaminobenzidine chromogen. Tissue samples from DS patients and controls were processed simultaneously. Double immunohistochemistry staining was performed after completion of the single staining protocol. Sections were sequentially incubated with 5% BSA for 10 min, monoclonal mouse anti-cytokeratin (clone AE1/AE3; DakoCytomation) diluted in PBS containing 0.1% BSA overnight at 4°C, and then with the Universal LSAB+ Kit/AP (DakoCytomation). Revelation was performed using the alkaline phosphatase permanent red chromogen. After the single or double protocol, the tissues were counterstained with Mayer's hematoxylin.

Quantification of stained cells and statistical analysis

Epithelial cells expressing nuclear AIRE or thymocytes expressing FOXP3 were identified and quantified using a light microscope. For 15 random areas of the medullary region of each thymus, the number of positive cells per square millimeter was determined using an integration graticule (Carl Zeiss 4740680000000 Netzmikrometer 12.5×) under ×400 magnification. Counting of positive cells was performed in a blinded fashion independently by two pathologists. Statistical analysis was initially performed with the Shapiro–Wilk normality test to determine whether the data were consistent with a normal distribution. Groups were compared using the two-tailed Student t test for unpaired data. Correlation coefficients were calculated using the Pearson correlation (r) test. The p values <0.05 were considered statistically significant. All statistical tests were performed using GraphPad Prism software.

Functional genomic studies

Obtention of thymic RNA. Fresh ex vivo explants from the thymuses of four DS and four control patients, all of them <2 y old, were collected at the operating room and immediately immersed into RNAlater RNA Stabilization Reagent (cat. no. 76104; Qiagen, Valencia, CA). RNA was extracted from tissue fragments using the RNeasy Lipid Tissue Mini Kit according to the manufacturer's instructions (cat. no. 74804; Qiagen).

Microarray hybridization and gene expression analysis. To determine gene expression profiles, 44 K DNA microarrays (cat. no. G4845A; Agilent Technologies, Santa Clara, CA) were used. The procedures for hybridization followed the protocols provided by the manufacturer's instructions (One-Color Microarray-Based Gene Quick Amp Labeling).

Expression analysis. The images were captured by the reader Agilent Bundle according to the parameters recommended for bioarrays and were

extracted by Agilent Feature Extraction software version 9.5.3. Among the 45,015 spots present in each array, only those with none or only one flag (i.e., low intensity, saturation, controls, etc.) were selected for analysis using the R software version 2.9.2 (R Development Core Team). By means of the TMEV software version 4.4.1 (21), we selected as differentially expressed transcripts those presenting a p value ≤0.05 (Student t test and adjusted Bonferroni correction) and fold variation of ±2. Hierarchical clustering was based on Pearson correlation and complete linkage. The significance analysis of microarrays (SAM) procedure (22) was used with a false discovery ratio of zero.

Transcriptional interaction analyses (gene ontology and network analysis). We used the FunNet software—based on the Gene Ontology Consortium (http://www.geneontology.org) and on the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg) genomic annotations—for performing the functional profiling of gene expression data and identifying the biological themes in which the differentially expressed genes are involved. Themes with significant relationship in the transcriptional expression space were associated to build transcriptional modules in a proximity network. A transcriptional interaction network, corresponding with the theme proximity network, was then obtained (http://www.funnet.info).

qPCR. Differential gene expression data were validated through qPCR. Specific primers for *AIRE* and five other selected genes (Table I) were designed using Primer-BLAST (Primer3 Input, version 0.4.0, and BLAST, available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All thymus RNA samples were amplified in triplicate. Amplification reactions were performed in a 25-μl final volume containing 1× SYBR Green mix (Quantitec SYBR Green PCR kit; Qiagen, Hilden, DE), 10 pmol of each primer, and 2 μl cDNA (1:10 dilution, synthesized from 1 μg of total RNA). Real-time PCR amplifications were performed in an Applied Biosystems StepOne Plus Real Time PCR System with StepOne software (Applied Biosystems, Foster City, CA) with the following cycling parameters: an initial hot start of 95°C for 15 min followed by 50 cycles of 95°C for 15 s and 60°C for 30 s. To normalize qPCR reactions, *GAPDH* was included as reference gene after checking that amplification curves for RNA samples obtained from five DS thymuses (four from our patients and one additional DS thymus sample kindly provided by Instituto de Medicina Integral de Pernambuco, Recife, PE, Brazil) and six control thymuses (four from our controls and two additional samples provided by Instituto de Medicina Integral de Pernambuco) yielded essentially the same results. Relative expression was determined by the relative standard curve method and presented as fold-change comparing DS versus control mean values.

Results

Quantification of AIRE-positive cells in the thymus

AIRE immunoreactivity was observed in nuclei of epithelial cells located in the medullary region, some of them around Hassall's corpuscles (Fig. 1A–D). AIRE-positive cells were marked as epithelial by costaining with a cytokeratin-specific Ab (Fig. 1E, 1F). The number of AIRE protein-expressing epithelial cells in thymic medulla of DS patients (70.48 ± 49.6 cells/mm^2; $n = 14$) was significantly lower ($p < 0.0001$) than that in the thymic medulla of the control group (154.70 ± 61.2 cells/mm^2; $n = 42$), these differences being more marked in infants (Fig. 2, Supplemental Fig. 1).

FOXP3-positive cells and correlation with AIRE expression

There was no significant difference in the numbers of FOXP3-positive thymocytes in thymic medulla of both groups: 824.4 ± 483.5 cells/mm^2 in DS ($n = 14$) and 617.0 ± 245.4 cells/mm^2 in controls ($n = 35$). Whereas the numbers of AIRE- and FOXP3-

Table I. Primers used in the qPCR assays with their sequences and respective product length

| Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Product Length (bp)
AIRE	ACCGGGTTTTCTTCCCAATA	AGAGACGCCCATGCAGACT	224
CDK6	TGGAGTGTTGGCTGCATATT	ACAGGGCACTGTAGGCAGAT	260
LMNB1	CTGGCGAAGATGTGAAGGTT	TCTGAGAAGGCTCTGCACTG	268
NUP93	TCAGGCACAACCTCTCAGAA	CCACAAAGCATGGCACTTAAT	254
PCNA	GAATTCCAGAACAGGAGTACAGC	TTCAGGTACCTCAGTGCAAAAG	258
TOP2A	GCTGCCCCAAAAGGAACTA	TAGGTTTCTTTGCCCGTACA	251
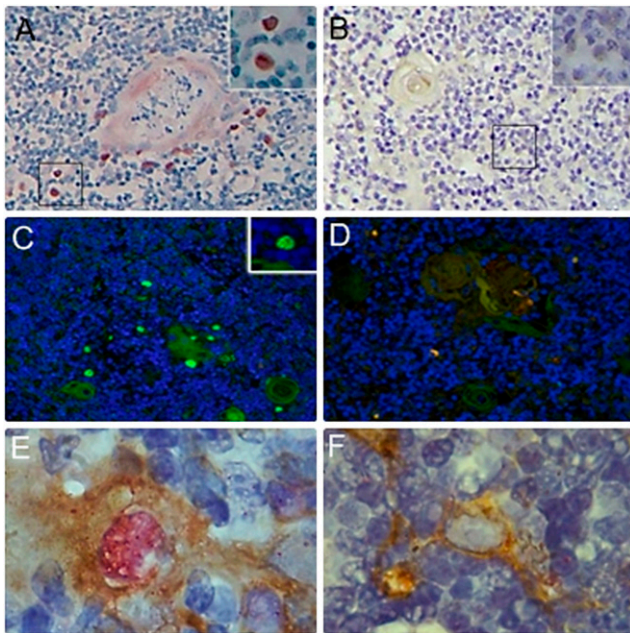
FIGURE 1. Representative illustrations of AIRE immunoreactivity (brown color) in thymic specimens from a control patient (*A*) and a DS patient (*B*). Nuclear AIRE is strongly stained in several cells of the thymic medullary region of control patients. *C* and *D*, Double-staining AIRE immunofluorescence (green color) in thymic specimens from a control patient (*C*) and a DS patient (*D*). *A–D*, Original magnification ×200 (*inset*, original magnification ×400). *E* and *F*, Double-staining immunohisto-chemistry for AIRE (brown color) and cytokeratin (reddish) showing an AIRE-positive (*E*) and an AIRE-negative (*F*) epithelial cell (original magnification ×1,000).

positive cells were not correlated in the control group ($r = 0.16$, $p = 0.35$, Fig. 3*A*), a significant positive correlation was found in DS ($r = 0.80$, $p = 0.0005$, Fig. 3*B*). Finally, no significant correlation was found between age and the numbers of either AIRE- or FOXP3-positive cells in both DS and control groups (data not shown).

Transcriptome profile analysis

We identified 21,940 valid thymic transcripts using the R program. Microarray data were deposited in the Gene Expression Omnibus public database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE23910. In the comparison of DS versus controls, a total of 1,238 differentially expressed transcripts were selected using permuted *t* test, adjusted *p* value ≤0.05 (Bonferroni), and
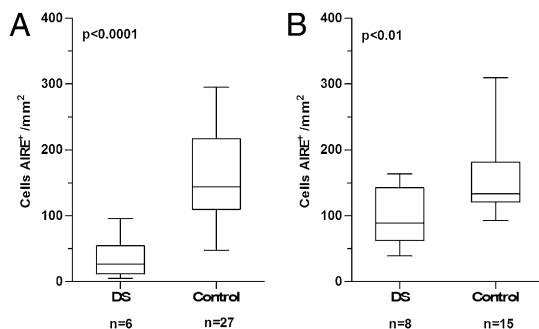
a fold of ±2. The SAM procedure (22) revealed 407 significantly hypoexpressed genes in the DS group (false discovery ratio = 0), whereas no hyperexpressed gene was observed. Hierarchical clustering showed complete separation between DS and controls (Supplemental Fig. 2). Overlap analysis (SAM and *t* test data), performed by means of the TMEV 4.4.1 program, yielded 156 hypoexpressed genes. The *AIRE* gene was not found significantly hypoexpressed in this analysis (fold of −1.87).

Network transcriptional analysis

This analysis was accomplished using SAM-selected differentially expressed genes and the FunNet software. The strength of the links between each pair of genes is given by Pearson's correlation co-efficient of expression profiles. From a total of 13,041 links, we selected the 654 links with a value above the third quartile (0.949).

A graphical representation of the functional gene profile according to gene ontology (GO) biological processes is shown in Fig. 4. The transcriptional domain coverage (GO categories) shows that the majority of the hypoexpressed genes belong to categories linked to cell cycle/cell proliferation or to immune response. Theme proximity network analysis (Fig. 5) confirms the picture revealed by GO categorization, as essentially two modules appear in this analysis, the first linked to cell division/proliferation and the second encompassing themes relevant to the immune response, such as Ag processing and presentation via MHC class II, T cell differentiation, selection, and activation, among others. The themes "male sex differentiation" and "male somatic sex determination" only encompass the X-linked androgen receptor gene (*AR*).

The picture showed in the theme proximity network was confirmed by the transcriptional interaction network analysis (Fig. 6), which depicts genes involved in immune function and in cell division/proliferation as the most frequent gene interactions. Table II shows selected relevant genes per category/function.

qPCR validation of DNA microarray data

Fig. 7 shows qPCR expression fold-changes comparing DS versus control samples for the genes (Table 1) *AIRE*, 2) *TOP2A, LMNB1*, and *NUP93* (*AIRE*-target genes), and 3) *CDK6* and *PCNA* (not modulated by *AIRE*). The results demonstrate downregulation of all these genes in DS thymus and corroborate DNA microarray expression values.

Discussion

This study revealed for the first time, to our knowledge, reduced thymic expression of a large set of genes that may constitute the basis for the molecular mechanisms underlying the immune disturbances characteristically seen in DS patients: thymus hypotrophy, higher propensity to develop organ-specific autoimmune disorders, and higher susceptibility to infection. Furthermore, DS thymus presents a pattern of widespread gene hypoexpression, which may well result in a hypofunctional thymic environment that characterizes the disease. Our data are in agreement with those of Aït Yahya-Graison et al. (23), who studied gene-expression variation of 136 genes located on chromosome 21 in DS lymphoblastoid cell lines and found that only 29% are expressed proportionally to the genomic-dosage imbalance, the other 71% having their expression "compensated" to normal or below-normal levels. Similar results were found by Prandini et al. (24) studying the gene-expression variation of DS lymphoblastoid and fibroblast cells. Notably, Sommer et al. (25) used serial analysis of gene expression methodology to study dysregulated genes in blood lymphocytes of DS children and found that none of the 30 mostly expressed tags was located on chromosome 21.

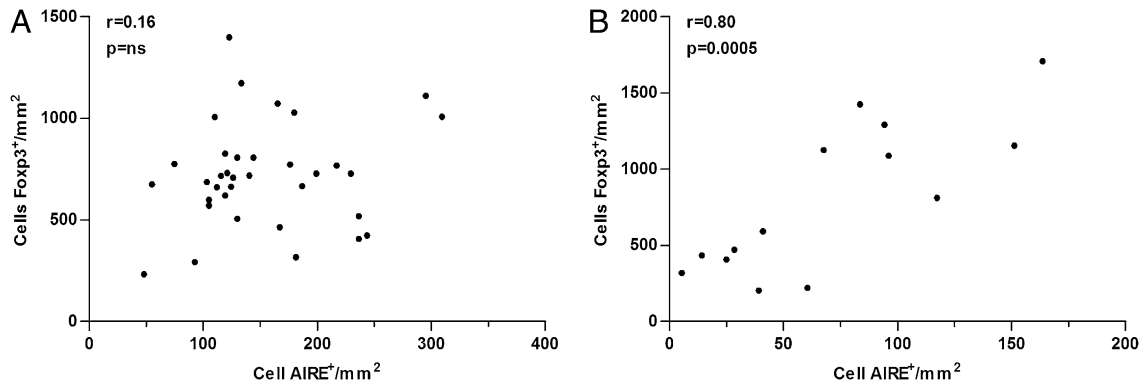**FIGURE 2.** Numbers of AIRE-positive cells/mm^2 in the thymic medullary region of DS and control groups. *A*, Infants (<1 y old). *B*, Children. DS patients from both age groups present significantly lower numbers of AIRE-positive cells/mm^2.

FIGURE 3. *A* and *B*, Correlation between the number of AIRE-positive cells/mm^2 and FOXP3-positive cells/mm^2 in thymic medula of control patients (*A*) and DS patients (*B*).

Decreased AIRE expression in DS

Prompted by some similarities between clinical and laboratory immunological features of DS and APECED (17, 26–31), we demonstrated reduced expression levels (RNA and protein) of AIRE—a chromosome 21 gene—in DS thymus. This finding corroborates those reported by Aït Yahya-Graison et al. (23), showing that AIRE is one of the chromosome 21 genes with reduced expression due to the trisomic imbalance. AIRE is a transcriptional regulator that commands the ectopic expression of tissue-specific Ags in thymic medullary epithelial cells (32–35), a critical mechanism for the selection of T cell repertoire emerging from thymus. AIRE function was largely revealed by APECED, a monogenic condition due to null or hypomorphic AIRE mutations (19, 20, 26–29, 31). It is surprising that a rather modest reduction in AIRE expression levels (a third or so) may

result in the severe clinical autoimmune disorder manifestations that characterize DS. It had previously been noted, however, that the presence of a functional AIRE allele in heterozygotic individuals does not prevent autoimmune disorder manifestations. An Italian APECED family was described in which all homozygous and heterozygous carriers of an AIRE mutation developed thyroiditis (36). More recently, this particular mutation (G228W) was cloned in a mouse model, and the resulting heterozygous mice developed autoimmunity (37). In short, together with these previous observations, our data indicate that "fine tuning" of thymic tissue-specific Ags expression is crucial in preventing autoimmune diseases. This may be particularly critical at early developmental times, as suggested by our observation of a greater AIRE expression deficit in the first year of life in DS (Fig. 2). It would appear that an "autoimmune condition" is set at these early periods, the "compensation" that occurs later having little effect in correcting it (38). Clearly, not all DS patients present autoimmune disorders, although most show reduced AIRE expression (Figs. 2, 3), suggesting that a pathogenic threshold may be influenced by other factors. Notably, *TOP2A*, an essential AIRE partner involved in chromatin remodeling and ectopic Ag expression, was also found hypoexpressed in DS thymus, as were three other AIRE partners (39), *LMNB1*, *NUP93*, and *PCNA*, as confirmed by qPCR (Fig. 7). Notably, *LMBB1* is positively stimulated in AIRE knockout mice (39), allowing the hypothesis that in DS, AIRE is just one among many hyporegulated genes.

Although some similarities between DS and APECED prompted us to investigate AIRE in DS (and, in fact, we now know that AIRE expression is altered in both conditions), they result from different pathophysiological mechanisms. In APECED, we have an *AIRE* mutation causing a severe impairment of gene function, whereas in DS, there is a moderate decrease in AIRE expression interplaying with a global thymic gene hypofunction caused by trisomy 21. Nonetheless, it is striking that both conditions show important commonalities regarding organ-specific autoimmune disorders, mainly endocrinopathies, as well as unique autoimmune Ab pattern (12, 17).

Impaired thymic function and development in DS

The molecular mechanisms regulating AIRE expression to lower-than-control levels in cases of trisomy 21 remain unclear, although a recent study demonstrated that a number of genes are subject to such "trisomic imbalance" (23). Notably, none of the significantly hypoexpressed genes (at the 2-fold level) that we found in this study is located on chromosome 21 (Table II). In contrast, chromosome 21 harbors five micro-RNA genes—miR-99a, let-7c, miR-125b-2, miR-155, and miR-802—that were already found to be overexpressed in the brain and heart of DS individuals (40) and
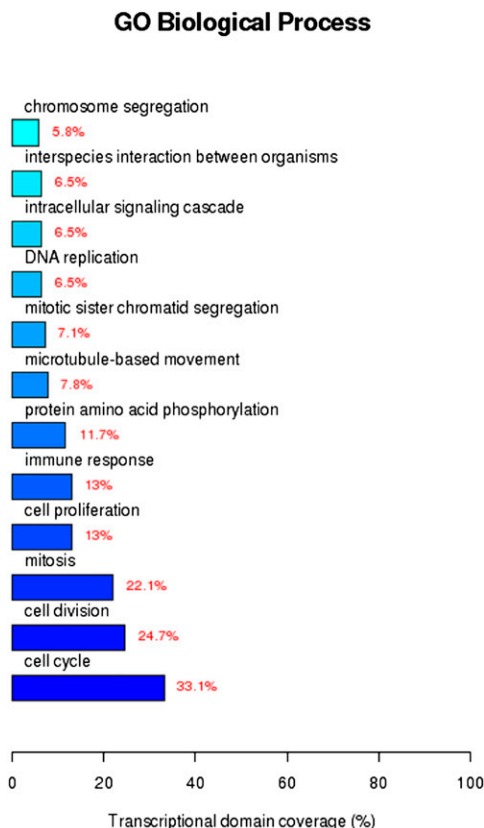

FIGURE 4. Graphical representation of GO biological processes functional profile based on the differentially expressed genes data set.
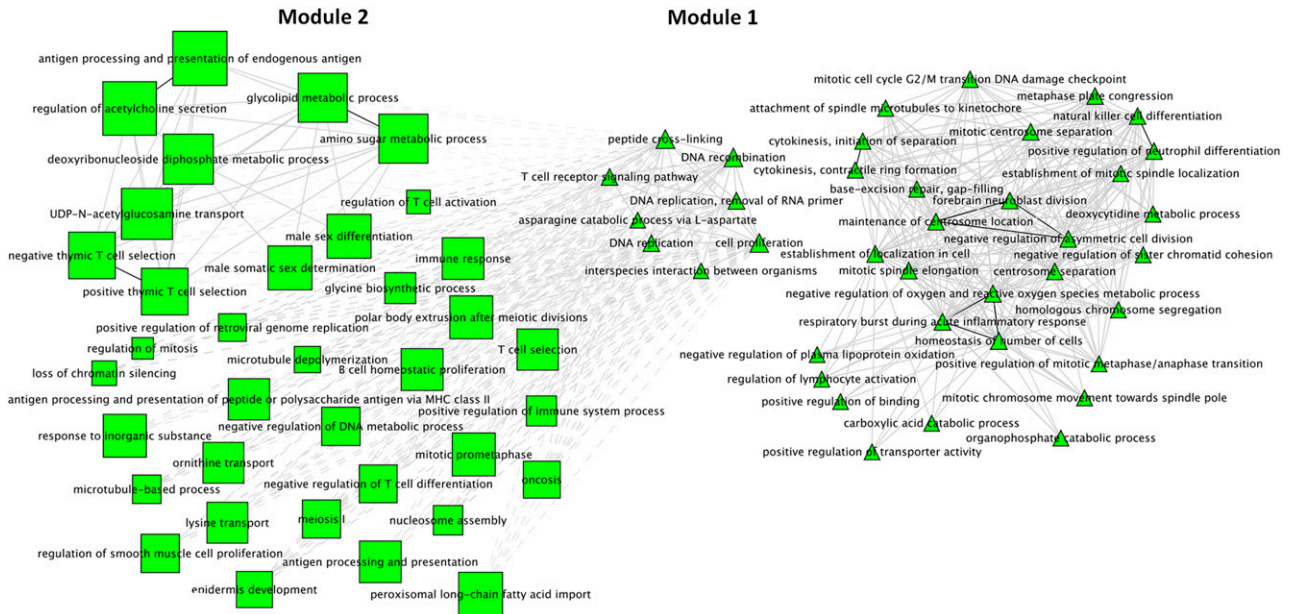
FIGURE 5. Theme proximity network built for GO biological process. Module 1 is represented by triangles and module 2 by squares.

may well exert regulatory effects on many of the genes described in this study. This is the case of miR-155, which regulates the development of regulatory T cells and the innate immune response through downregulation of *SOCS1* (41), and of miR-125-b and let-7c, which regulate macrophage responses to various stimuli (42, 43).

Yet, our global gene expression and transcriptional network analyses demonstrated deficient expression of many other genes in DS thymus. Notably, a number of these genes are known to reg-

ulate biological processes related to the development/activation of T cells and to the establishment of central tolerance (Figs. 4–6 and Table II): 1) Ag processing and presentation of Ag via MHC class II (*ERAP2, CD1D, HLA-DQB1, HLA-DRB3, CD1A, CD1B, CD1C*); 2) thymic T cell selection (*CD3D, CD74, CD1D, CD3E*), 3) T cell activation (*LAT*). It follows that susceptibility to organ-specific autoimmune disorder in DS may not be the consequence of deficient *AIRE* expression only, but owe as well to the reduced expression of other genes involved in critical thymic functions.

FIGURE 6. Transcriptional interaction network corresponding with the theme proximity network related to GO biological process. Colored circles indicate predominant gene function.
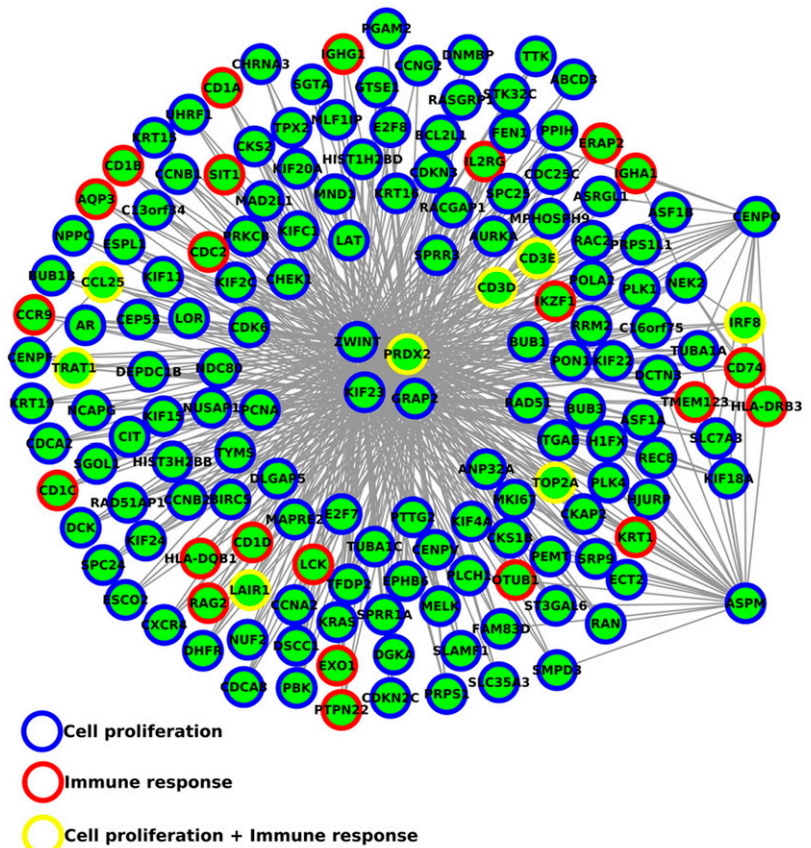
Table II. Selected relevant genes hypoexpressed in DS thymus displayed by category and function

Gene Symbol	Gene Product	Chromosomal Location	Biological Process (Module 1)	Biological Process (Module 2)
KIF23	Kinesin-like protein family	15q23	DNA metabolic process; mitosis; cell division; cell cycle, DNA repair	Microtubule-based process
ZWINT	A protein involved in kinetochore function	10q21-q22	Chromosome organization; spindle organization, mitosis; cell division; cell cycle; chromosome segregation, etc.	Microtubule-based process
GRAP2	This gene encodes a member of the GRB2/Sem5/Drk family. This member is an adapter-like protein involved in leukocyte-specific protein-tyrosine kinase signaling.	22q13.2	Cell division, mitosis, Ras protein signal transduction, intracellular signaling cascade	—
PRDX2	Peroxiredoxin family of antioxidant enzymes	19p13.2	Lymphocyte activation; cell proliferation; cell division; regulation of hydrogen peroxide metabolic process; thymus development; T cell differentiation; regulation of lymphocyte activation; homeostasis of number of cells	Regulation of T cell activation; immune response; negative regulation of T cell differentiation
PRSS16	Serine protease expressed exclusively in the thymus	6p21	Nucleotide binding	—
IL2RG	IL-2R, γ	Xq13.1	Lymphocyte activation; cell division; mitosis; interspecies interaction between organism; T cell activation	Regulation of T cell activation; positive regulation of immune system process; immune response
RAG2	This gene encodes a protein that is involved in the initiation of V(D)J recombination during B and T cell development	11p3	DNA metabolic process, cell proliferation, cell division, mitosis, T cell differentiation, DNA recombination	B cell homeostatic proliferation, immune response
SH2D1A	Role in the bidirectional stimulation of T and B cells	Xq25-q26	Mitosis	Positive regulation of immune system process
CD3D	Part of the TCR–CD3 complex	11q23	Lymphocyte activation, cell division, mitosis, T cell differentiation	Positive thymic T cell selection, T cell selection
CD3E	CD3-ε polypeptide	11q23	Cell proliferation; protein amino acid phosphorylation; lymphocyte activation; T cell differentiation	Negative thymic T cell selection; T cell selection
HLA-DRB3	HLA-DRB3 belongs to the HLA class II β-chain paralogs. This class II molecule is a heterodimer consisting of an α (DRA) and a β (DRB) chain, both anchored in the membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins.	6p21.3	Mitosis	Immune response, Ag processing and presentation
HLA-DQB1	HLA-DQB1 belongs to the HLA class II β-chain paralogs	6p21.3	Cell division, mitosis	Ag processing and presentation of peptide or polysaccharide Ag via MHC class II, Ag processing and presentation
CD1A	CD1 family of transmembrane glycoproteins	1q22-q23	Cell division, mitosis	Ag processing and presentation
CD1B	CD1 family of transmembrane glycoproteins	1q22-q23	Cell division	Immune response; Ag processing and presentation
CD1C	CD1 family of transmembrane glycoproteins	1q22-q23	Mitosis; cell division	Immune response; Ag processing and presentation
CD1D	CD1 family of transmembrane glycoproteins	1q22-q23	Lymphocyte activation; mitosis; interspecies interaction between organisms; T cell differentiation	Ag processing and presentation of endogenous Ag; immune response; T cell selection; Ag processing and presentation

(Table continues)

Table II. (*Continued*)

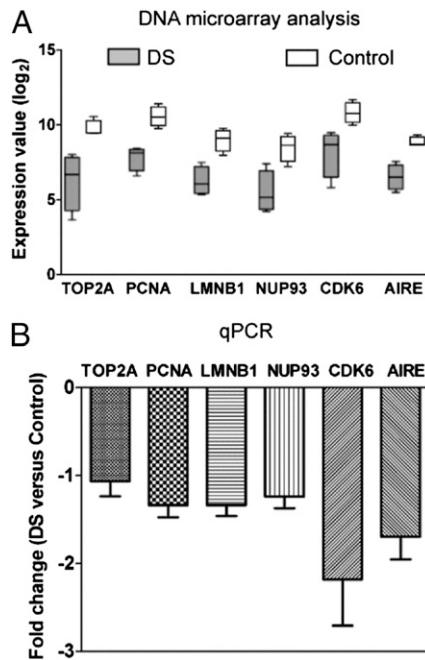| Gene Symbol	Gene Product	Chromosomal Location	Biological Process (Module 1)	Biological Process (Module 2)
CD74	CD74 molecule, MHC class II invariant chain	5q32	Mitosis, intracellular signaling cascade, protein amino acid phosphorylation, lymphocyte activation, cell proliferation, cell division	Ag processing and presentation of endogenous Ag, positive regulation of immune system process, positive regulation of immune system process, regulation of T cell activation, negative thymic T cell selection, immune response, positive thymic T cell selection, T cell selection
TOP2A	Topoisomerase (DNA) II α. This gene is an AIRE partner and encodes a DNA topoisomerase that controls and alters the topologic states of DNA during transcription.	17q21-q22	DNA replication, intracellular signaling cascade	Positive regulation of retroviral genome replication
LCK	Protein tyrosine kinases	1p34.3	Protein amino acid phosphorylation; cell division; mitosis; positive regulation of TCR signaling pathway; interspecies interaction between organisms; TCR signaling pathway	Positive regulation of immune system process; regulation of T cell activation; immune response
LAT	Linker for activation of T cells	16p11.2	Lymphocyte activation; cell division; mitosis; RAS protein signal transduction; regulation of lymphocyte activation; intracellular signaling cascade	Regulation of T cell activation; immune response
ERAP2	ERAP1 (MIM 606832) and LRAP to trim precursors to antigenic peptides in the endoplasmic reticulum	5q15	Cell division, mitosis	Ag processing and presentation of endogenous Ag, immune response
IKZF1	Family zinc finger 1	7p13-p11.1	T cell differentiation, regulation of lymphocyte activation, lymphocyte activation	Positive regulation of immune system process, regulation of T cell activation
SLAMF1	Signaling lymphocytic activation molecule family member 1	1q22-q23	Interspecies interaction between organisms, lymphocyte activation, cell proliferation, cell division, mitosis	—
CXCR4	CXCR specific for stromal cell-derived factor-1	2q21	Mitosis, interspecies interaction between organisms, intracellular signaling cascade, cell division	Immune response
CHEK1	CHK1 checkpoint homolog (*S. pombe*)	11q24-q24	Cell proliferation, cell division, mitosis, meiosis, cell cycle	Meiosis
FEN1	DNA repair and processes the 5′ ends of Okazaki fragments in lagging strand DNA synthesis	11q12	Cell division, mitosis	—
CCNB2	Cyclin family	15q22.2	Cell division, mitosis, cell cycle, thymus development	—
MLF1IP	Specialized chromatin domain	4q35.1	Mitosis, interspecies interaction between organisms, cell division	—
E2F7	Plays an essential role in the regulation of cell cycle progression	12q21.2	Cell proliferation	—

—, Not present in Module 2.

FIGURE 7. qPCR validation of DNA microarray data. *A*, Boxplots comparing the DNA microarray expression values of six selected genes in DS (gray) and control (white) samples. *B*, qPCR expression fold-changes comparing DS with control samples for the same genes showing downregulation in DS. qPCR was performed using RNA samples from five DS patients and six control patients.

Altogether, our data reinforce the pivotal role of defective central tolerance in the pathogenesis of DS autoimmune disorders. Thus, the thymus in DS individuals had been reported to be smaller and hypocellular, even in infants, containing a decreased proportion of phenotypically mature TCR-αβ$^+$ thymocytes (6). The number of TRECs and the size of T cell subpopulations (CD4$^+$, CD8$^+$, CD4$^+$ CD45$^+$RA cells) in the peripheral blood of DS individuals have been described as reduced at various age groups (4, 6, 7). It was also demonstrated that DS patients present low naive T cell numbers (44). Additionally, DS individuals fail to show the normal, notorious expansion of circulating lymphocyte numbers in the first months of life (45). Our finding that several genes related to cell proliferation (Figs. 4–6 and Table II) are hypofunctional in DS (1) cell cycle regulation [*E2F7*]; 2) cell proliferation [*ZWINT, KIF23, CHEK1*]; 3) DNA replication [*FEN1*]; 4) homeostasis of number of cells [*PRDX2*], and 5) thymus development [*PRDX2, IKZF1, CCNB2, CDK6*]) may be causally related to DS immune phenotype (i.e., thymus hypotrophy and hypocellularity). It is interesting to note that *CDK6* was recently found to be essential for thymocyte development (46).

In contrast with the scenario described above, augmented numbers of FOXP3$^+$CD25$^+$ natural regulatory T cells were observed in DS peripheral blood (7), which is in accordance with our data showing normal numbers of FOXP3-positive cells in DS thymus (Fig. 3).

Global gene profiles and transcriptional network analyses presented in this study may also help to understand the higher susceptibility to infections systematically described in DS (5, 6, 12). Thus, mutations of genes that were found hypoexpressed in DS thymus have been associated with severe primary immunodeficiencies (47): *IL2RG* (X-linked SCID), *RAG2, CD3D, CD3E* (SCID), and *SH2D1A* (X-linked lymphoproliferative syndrome). Notably, milder immunodeficiency forms, in which autoimmune manifestations are frequently part of the clinical picture, were

also associated with mutations in some of these genes, including *IL2RG* and *RAG* (48). Furthermore, other hypoexpressed genes in DS thymus are involved in other biological processes that are also relevant for resistance to infections: 1) positive regulation of neutrophil differentiation (*IKZF1*), 2) NK cell differentiation (*IKZF1*), 3) respiratory burst during acute inflammatory response (*PRDX2*), 4) negative regulation of oxygen and reactive oxygen species metabolic process (*PRDX*2), among others. Although many such processes are unlikely to occur inside the thymus, hypoexpression due to trisomic imbalance may well result in deficient immune functions in the periphery. This may be the case of the genes categorized as "interspecies interaction between organisms" (i.e., *IL2RG, KRT19, LCK, RAN, SGTA, SLAMF1, CXCR4, MLF1IP, CD1D*).

It should also be noted that the genes with more interactions (hubs) in the transcriptional network analysis (Fig. 6) were 1) *PRDX2*, which codes for a member of the peroxiredoxin family of antioxidant enzymes involved in T cell antiviral activity (49) and in thymus development, and was described as hypoexpressed in fetal DS brain (50); 2) *GRAP2*, which codes for an adapter-like protein involved in leukocyte-specific protein-tyrosine kinase signaling (51); 3) *ZWINT*, involved in kinetochore function (52); and 4) *KIF23*, which codes for a kinesin-like protein family involved in chromosome movement during cell division (53).

DS: a primary immunodeficiency?

The current study 1) contributes to the understanding of thymic hypotrophy in DS patients, 2) demonstrates its association with reduced expression of critical genes, probably derived from trisomic imbalance, and 3) strongly suggests that DS typical immune malfunction is owed to impaired central tolerance, possibly due to both decreased AIRE expression and global thymic hypofunction. Thus, our results are in general agreement with the recent proposal by Kusters et al. (6) that "the immune system in DS is intrinsically deficient from the very beginning, and not simply another victim of a generalized process of precocious aging," as hypothesized by others (7, 8, 54, 55). Altogether, our data indicate that DS is indeed a primary, rather than a secondary, immunodeficiency, contrary to what is largely accepted (47). It would seem, therefore, that DS should well be considered as a non-monogenic primary immunodeficiency (PID). It has largely been recognized that most PIDs are monogenic disorders (47); however, there exist good examples of polygenic PIDs (such as DiGeorge syndrome) that are caused by a deletion encompassing several loci on chromosome 22q11.2 (56).

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SUPPLEMENTAL DATA

FIGURE S1: Box plots refer to the number of AIRE-positive cells in individual areas from each thymus in control and DS groups. Counting of AIRE-positive cells was performed in a blinded fashion without any significant intra or interobserver variation.
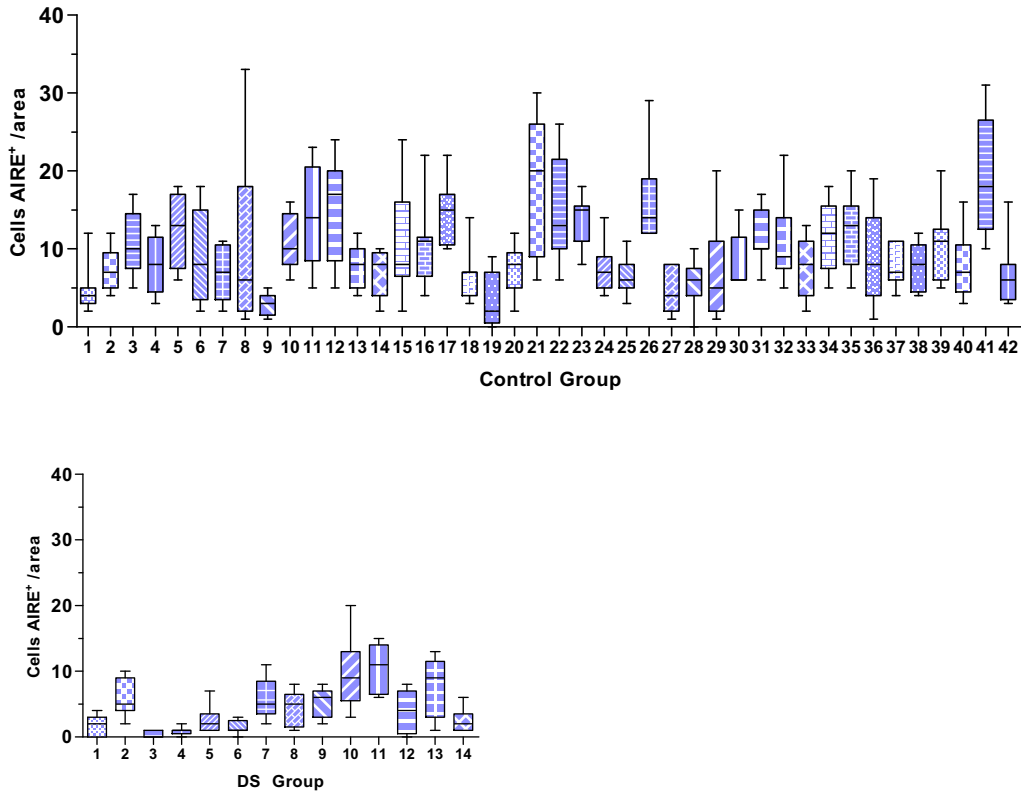
FIGURE S2: Hierarchical clustering for 407 differentially expressed transcripts found in DS thymuses. Red tones indicate hyperexpression and green tones indicate hypoexpression. In the top line green indicates controls and blue indicates patient's samples.