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Decreased AIRE Expression and Global Thymic Hypofunction in Down Syndrome

Flavia A. Lima,*1 Carlos A. Moreira-Filho,∗† Patrícia L. Ramos,* Helena Brentani,†
Leandro de A. Lima,‡ Magaly Arrais,§ Luiz C. Bento-de-Souza,§ Luciana Bento-de-Souza,§
Maria I. Duarte,§ Antonio Coutinho,† and Magda Carneiro-Sampaio*∗

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/mm² in DS versus 154.70 ± 61.16 AIRE-positive cells/mm² in controls (p < 0.0001), and quantitative PCR as well as DNA microarray data confirmed those results. The number of FOXP3-positive cells/mm² was equivalent in both groups. Thymus transcriptome analysis showed 407 genes significantly hypoxpressed 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 hypoxpressed 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.

The online version of this article contains supplemental material.

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
complications; another developed Hashimoto thyroiditis at 3 y old. Control
samples (42 thyromes) 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 depara-
affinized and rehydrated through graded alcohols to water. For antigenic
retrieval, sections were microwave-treated at 800 W in 10 m M 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% H2O2 for 10 min at room temperature. Unspecific
binding was blocked by incubation with 10% normal goat serum. Sections
were incubated overnight at 4°C with a polyclonal rabbit anti-AIRE serum
(sco-33188; Santa Cruz Bio-
technology, 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), 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 counter-
stained 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 μm) under x400 magnifica-
tion. Counting of positive cells was performed in a blinded fashion
independently by two pathologists. Statistical analysis was initially per-
formed 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’s t test. The 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 thyromes of four
DS and four control patients, all of them <2 y old, were collected at the
opening room and immediately frozen in RNA later 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. G4454A; Agilent
Technologies, Santa Clara, CA) were used. The procedures for hybrid-
ization followed the protocols provided by the manufacturer’s instructions
(One-Color Microarray-Based Gene Quick Amp Labeling). ARIE

**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.genontology.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 ex-
pression space were associated to build transcriptional modules in a prox-
imity network. A transcriptional interaction network, corresponding with
the theme proximity network, was then obtained (http://www.funnel.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,
RNA samples were amplified in triplicate. Amplification reactions were
performed in a 25-μl final volume containing 1× SYBR Green mix
(Quantitect 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 Bio-
systems StepOne Plus Real Time PCR System with StepOne software
(a) (http://www.appliedbiosystems.com) with the following cycling para-
eters: 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 because after amplification curves that
amplification curves for RNA samples obtained from five DS thyromes
(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 thyromes (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. 1 A–D). AIRE-positive cells were marked as ep-
thelial by costaining with a cytokeratin-specific Ab (Fig. 1 E, 1 F).
The number of AIRE protein-expressing epithelial cells in thymic
medulla of DS patients (70.48 ± 49.6 cells/mm²; n = 14) was significantly
lower (p < 0.0001) than that in the thymic medulla of the
control group (154.70 ± 61.2 cells/mm²; 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/mm² in DS (n = 14) and 617.0 ± 245.4 cells/mm² in
controls (n = 35). Whereas the numbers of AIRE- and FOXP3-

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>Product Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIRE</td>
<td>ACCGGGTATTCTTCTCCAAATA</td>
<td>AGAGACCCCATGTCAGACT</td>
<td>224</td>
</tr>
<tr>
<td>CDK6</td>
<td>TGGAAGATGCTGGTCATATT</td>
<td>ACAGGGCACTGTAGCACAGT</td>
<td>260</td>
</tr>
<tr>
<td>LMNB1</td>
<td>CGCCGCAAAGATGTAGAATT</td>
<td>TGGAGAACGCTTGCACCTG</td>
<td>268</td>
</tr>
<tr>
<td>NUP93</td>
<td>TCAAGCCACCACCTCCTGAGA</td>
<td>CCACAAAGCTAGGACCTTATA</td>
<td>254</td>
</tr>
<tr>
<td>PCNA</td>
<td>GAATCCTCGAAGAGGATGACG</td>
<td>TTCAGGTCTCCTGTTGAAA</td>
<td>258</td>
</tr>
<tr>
<td>TOP2A</td>
<td>GCTGCCAAAGAAGGACTA</td>
<td>TAGGGTTCTTCCTGGCCCTACA</td>
<td>251</td>
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</tbody>
</table>
positive cells were not correlated in the control group ($r = 0.16$, $p = 0.35$, Fig. 3A), a significant positive correlation was found in DS ($r = 0.80$, $p = 0.0005$, Fig. 3B). 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 $\leq 0.05$ (Bonferroni), and a fold of $\pm 2$. 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 coefficient 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 Ait 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.
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 Aıt 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, LMNB1 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

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**FIGURE 3.** A and B, Correlation between the number of AIRE-positive cells/mm² and FOXP3-positive cells/mm² in thymic medula of control patients (A) and DS patients (B).

**FIGURE 4.** Graphical representation of GO biological processes functional profile based on the differentially expressed genes data set.
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 regulate 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.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Product</th>
<th>Chromosomal Location</th>
<th>Biological Process (Module 1)</th>
<th>Biological Process (Module 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIF23</td>
<td>Kinesin-like protein family</td>
<td>15q23</td>
<td>DNA metabolic process; mitosis; cell division; cell cycle; DNA repair</td>
<td>Microtubule-based process</td>
</tr>
<tr>
<td>ZWINT</td>
<td>A protein involved in kinetochore function</td>
<td>10q21-q22</td>
<td>Chromosome organization; spindle organization; mitosis; cell division; cell cycle; chromosome segregation; etc.</td>
<td>Microtubule-based process</td>
</tr>
<tr>
<td>GRAP2</td>
<td>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.</td>
<td>22q13.2</td>
<td>Cell division, mitosis, Ras protein signal transduction, intracellular signaling cascade</td>
<td>—</td>
</tr>
<tr>
<td>PRDX2</td>
<td>Peroxiredoxin family of antioxidant enzymes</td>
<td>19p13.2</td>
<td>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</td>
<td>Regulation of T cell activation; immune response; negative regulation of T cell differentiation</td>
</tr>
<tr>
<td>PRSS16</td>
<td>Serine protease expressed exclusively in the thymus</td>
<td>6p21</td>
<td>Lymphocyte activation; cell division; mitosis; interspecies interaction between organism; T cell activation</td>
<td>—</td>
</tr>
<tr>
<td>IL2RG</td>
<td>IL-2R, γ</td>
<td>Xq13.1</td>
<td>Lymphocyte activation; cell division; mitosis; interspecies interaction between organism; T cell activation</td>
<td>Regulation of T cell activation; positive regulation of immune system process; immune response</td>
</tr>
<tr>
<td>RAG2</td>
<td>This gene encodes a protein that is involved in the initiation of V(D)J recombination during B and T cell development</td>
<td>11p3</td>
<td>DNA metabolic process, cell proliferation, cell division, mitosis, T cell differentiation, DNA recombination</td>
<td>B cell homeostatic proliferation, immune response</td>
</tr>
<tr>
<td>SH2D1A</td>
<td>Role in the bidirectional stimulation of T and B cells</td>
<td>Xq25-q26</td>
<td>Lymphocyte activation; cell division, mitosis, T cell differentiation</td>
<td>Positive regulation of immune system process</td>
</tr>
<tr>
<td>CD3D</td>
<td>Part of the TCR-CD3 complex</td>
<td>11q23</td>
<td>Lymphocyte activation, cell division, mitosis, T cell differentiation</td>
<td>Positive thymic T cell selection, T cell selection</td>
</tr>
<tr>
<td>CD3E</td>
<td>CD3-ε polypeptide</td>
<td>11q23</td>
<td>Cell proliferation; protein amino acid phosphorylation; lymphocyte activation; T cell differentiation</td>
<td>Negative thymic T cell selection; T cell selection</td>
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<td>HLA-DRB3</td>
<td>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.</td>
<td>6p21.3</td>
<td>Lymphocyte activation; cell proliferation; cell division; mitosis; interspecies interaction between organisms; T cell differentiation</td>
<td>Immune response, Ag processing and presentation</td>
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<tr>
<td>HLA-DQB1</td>
<td>HLA-DQB1 belongs to the HLA class II β-chain paralogs</td>
<td>6p21.3</td>
<td>Cell division, mitosis</td>
<td>Ag processing and presentation of peptide or polysaccharide Ag via MHC class II, Ag processing and presentation</td>
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<tr>
<td>CD1A</td>
<td>CD1 family of transmembrane glycoproteins</td>
<td>1q22.2-q23</td>
<td>Cell division, mitosis</td>
<td>—</td>
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<tr>
<td>CD1B</td>
<td>CD1 family of transmembrane glycoproteins</td>
<td>1q22.2-q23</td>
<td>Cell division</td>
<td>Ag processing and presentation</td>
</tr>
<tr>
<td>CD1C</td>
<td>CD1 family of transmembrane glycoproteins</td>
<td>1q22.2-q23</td>
<td>Mitosis; cell division</td>
<td>Immune response; Ag processing and presentation</td>
</tr>
<tr>
<td>CD1D</td>
<td>CD1 family of transmembrane glycoproteins</td>
<td>1q22.2-q23</td>
<td>Lymphocyte activation, mitosis; interspecies interaction between organisms; T cell differentiation</td>
<td>Ag processing and presentation of endogenous Ag; immune response; T cell selection; Ag processing and presentation</td>
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(Table continues)
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Product</th>
<th>Chromosomal Location</th>
<th>Biological Process (Module 1)</th>
<th>Biological Process (Module 2)</th>
</tr>
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<tr>
<td>CD74</td>
<td>CD74 molecule, MHC class II invariant chain</td>
<td>5q32</td>
<td>Mitosis, intracellular signaling cascade, protein amino acid phosphorylation, lymphocyte activation, cell proliferation, cell division</td>
<td>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</td>
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<tr>
<td>TOP2A</td>
<td>Topoisomerase (DNA) II alpha. This gene is an AIRE partner and encodes a DNA topoisomerase that controls and alters the topologic states of DNA during transcription.</td>
<td>17q21-q22</td>
<td>DNA replication, intracellular signaling cascade</td>
<td>Positive regulation of retroviral genome replication</td>
</tr>
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<td>LCK</td>
<td>Protein tyrosine kinases</td>
<td>1p34.3</td>
<td>Protein amino acid phosphorylation; cell division; mitosis; positive regulation of TCR signaling pathway; interspecies interaction between organisms; TCR signaling pathway</td>
<td>Positive regulation of immune system process; regulation of T cell activation; immune response</td>
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<td>LAT</td>
<td>Linker for activation of T cells</td>
<td>16p11.2</td>
<td>Lymphocyte activation; cell division; mitosis; RAS protein signal transduction; regulation of lymphocyte activation; intracellular signaling cascade</td>
<td>Regulation of T cell activation; immune response</td>
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<td>ERAP2</td>
<td>ERAP1 (MIM 606832) and LRAP to trim precursors to antigenic peptides in the endoplasmic reticulum</td>
<td>5q15</td>
<td>Cell division, mitosis</td>
<td>Ag processing and presentation of endogenous Ag, immune response</td>
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<td>IKZF1</td>
<td>Family zinc finger 1</td>
<td>7p13-p11.1</td>
<td>T cell differentiation, regulation of lymphocyte activation, lymphocyte activation</td>
<td>Positive regulation of immune system process, regulation of T cell activation</td>
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<td>SLAMF1</td>
<td>Signaling lymphocytic activation molecule family member 1</td>
<td>1q22-q23</td>
<td>Interspecies interaction between organisms, lymphocyte activation, cell proliferation, cell division, mitosis</td>
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<td>CXCR4</td>
<td>CXCR specific for stromal cell-derived factor-1</td>
<td>2q21</td>
<td>Mitosis, interspecies interaction between organisms, intracellular signaling cascade, cell division</td>
<td>Immune response</td>
</tr>
<tr>
<td>CHEK1</td>
<td>CHK1 checkpoint homolog (S. pombe)</td>
<td>11q24-q24</td>
<td>Cell proliferation, cell division, mitosis, meiosis, cell cycle</td>
<td>Meiosis</td>
</tr>
<tr>
<td>FEN1</td>
<td>DNA repair and processes the 5' ends of Okazaki fragments in lagging strand DNA synthesis</td>
<td>11q12</td>
<td>Cell division, mitosis</td>
<td>—</td>
</tr>
<tr>
<td>CCNB1</td>
<td>Cyclin family</td>
<td>15q22.2</td>
<td>Cell division, mitosis, cell cycle, thymus development</td>
<td>—</td>
</tr>
<tr>
<td>MLF1IP</td>
<td>Specialized chromatin domain</td>
<td>4q35.1</td>
<td>Mitosis, interspecies interaction between organisms, cell division</td>
<td>—</td>
</tr>
<tr>
<td>E2F7</td>
<td>Plays an essential role in the regulation of cell cycle progression</td>
<td>12q21.2</td>
<td>Cell proliferation</td>
<td>—</td>
</tr>
</tbody>
</table>

— Not present in Module 2.
TRECs and the size of T cell subpopulations (CD4+, CD8+, CD4+ deficiencies (47): DS thymus have been associated with severe primary immunodeficiency systematically described in DS (5, 6, 12). Thus, mutations of genes that were found hypoexpressed in DS (1) cell cycle regulation ([E2F7]); 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 (PRDX2), 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, LC5, RAN, SGTA, SLAMF1, CXC4, 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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Disclosures

The authors have no financial conflicts of interest.

References


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.