

BRIEF REPORT

Effect of CD3 δ Deficiency on Maturation of α/β and γ/δ T-Cell Lineages in Severe Combined Immunodeficiency

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THE T-CELL-RECEPTOR COMPLEX CONSISTS OF THE α AND β OR γ AND δ variant chains, paired as mutually exclusive heterodimers in association with the invariant chains CD3 γ , δ , ϵ , and ζ . T cells with α and β chains are referred to as α/β T cells, and those with γ and δ chains are called γ/δ T cells. During development, the CD3 protein complex plays an important part in the transition of thymocytes from CD4-CD8- double-negative immature precursors to a CD4+CD8+ double-positive stage and finally to the mature CD4+CD8- or CD4-CD8+ single-positive T cell.¹⁻⁵ Selective deficiency of CD3 component γ , δ , ϵ , or ζ in mice, achieved by gene knockout, causes mild-to-severe, although incomplete, blockage of T-cell development.⁶⁻¹⁰ Similarly, CD3 γ or CD3 ϵ deficiency in humans brings about a partial arrest of T-cell maturation and only moderate immunodeficiency.^{11,12}

We report a novel defect in the CD3 δ gene in three members of a kindred with a form of severe combined immunodeficiency (SCID) characterized by the absence of T cells but normal numbers of B cells (T-B+ SCID). These three patients had an early arrest in T-cell development, with a nearly complete absence of circulating mature T cells and a complete lack of γ/δ T cells. Our results suggest that, unlike CD3 ϵ and CD3 γ , CD3 δ is essential for T-cell development.

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CASE REPORT

We studied a kindred of Mennonite descent that shared multiple consanguineous links across several generations. Three patients with SCID were identified in this family. SCID was diagnosed in Patient 1 immediately after birth, after an examination performed because of previous cases in the family (Patients 2 and 3). She subsequently underwent bone marrow transplantation and is alive and well, with full immune reconstitution, three years later. Patient 2, a male cousin of Patient 1, was admitted at the age of two months because of fever, tachypnea, and tachycardia. Rapidly developing respiratory arrest required assisted ventilation, and he died of multiorgan failure. Adenovirus was identified in stool, urine, and bronchial secretions.

Patient 3, a male cousin of Patients 1 and 2, was well and thriving until two and a half months of age, when chronic diarrhea developed. At three and a half months of age, the patient was admitted with respiratory distress, lethargy, and jaundice. On examination, he was noted to have hepatomegaly, and liver-function tests were markedly abnormal. He was transferred from another hospital with increased respiratory distress and died 12 hours later from rapidly developing refractory hypotension, liver failure, pulmonary hemorrhage, disseminated intravascular coagulopathy, and hemorrhagic shock. Cytomegalovirus was identified in multiple tissues obtained at autopsy.

Flow-cytometric analyses of peripheral-blood lymphocytes from these patients showed a slight reduction in total lymphocyte counts in Patients 1 and 2 and a marked

reduction in Patient 3 (Table 1). The numbers of circulating mature CD3+ T cells were extremely low (3 to 7 cells per cubic millimeter), whereas CD4+ or CD8+ T cells were undetectable. In contrast, the number of B cells, as determined by staining for CD20, was either normal (in Patients 1 and 3) or increased (in Patient 2). The number of natural killer cells, as determined by staining for CD56, was normal in all patients.

An extreme deficiency of T cells in T-B+ SCID (with or without natural killer cells) is frequently associated with a small, dysplastic thymus, which is barely detectable by radiography or ultrasonography.¹³ In contrast, all three patients had a nearly normal sized thymus shadow on chest radiography. Analysis of thymus-gland tissue obtained by biopsy in Patient 1 and at autopsy from Patient 3 and stained with hematoxylin and eosin revealed preserved lobular structures with moderate populations

of T-cell precursors. However, typical intralobar corticomedullary distinctions and Hassall's corpuscles were absent (not shown).

METHODS

ASSAYS OF PHENOTYPE AND FUNCTION OF PERIPHERAL-BLOOD LYMPHOCYTES

Cell-surface markers of peripheral-blood lymphocytes were determined by immunofluorescence antibody staining and flow cytometry (Epics V, Coulter Electronics) with antibodies purchased from Coulter Diagnostics. In vitro lymphocyte proliferation induced by phytohemagglutinin was assayed by standard means.

ANALYSIS OF THYMIC TISSUE

After written informed consent was obtained from the parents of the patients and control infants, samples of thymus tissue obtained by biopsy (Patient 1), at autopsy (Patient 3), and from four infants undergoing cardiac surgery were obtained for analysis. Then, 4- μ m serial sections of frozen thymus tissue were mounted on glass slides, air-dried, and stained with hematoxylin and eosin or with specific antibodies raised against the various T-cell receptor, CD3, CD4, and CD8 chains.

WESTERN BLOTTING

Thymocytes obtained from Patient 1 or from normal thymus were isolated by centrifugation and lysed in 50 μ l of lysis buffer (20 mM TRIS [pH 7.4], 150 mM sodium chloride, 1 percent Igepal CA-630, 5 mM EDTA, 2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonylfluoride), incubated on ice for 15 minutes, and then centrifuged for 10 minutes at 12,000 \times g. The proteins in the supernatant were analyzed by a standard Western blotting technique with the use of antibodies against T-cell receptor α (SC-9100), T-cell receptor β (SC-5277), T-cell receptor γ (SC-9854), T-cell receptor δ (SC-1578), CD3 γ (SC-1125), CD3 δ (SC-1128), CD3 ϵ (SC-1179), CD3 ζ (SC-1239), guanine nucleotide-binding protein α -inhibitory subunit 3 (G α i-3, SC-262), CD4 (SC-7219), CD8 α (SC-7970), and CD3 β (SC-9147), all purchased from Santa Cruz Biotechnology.

PREPARATION OF RNA, GENOMIC DNA, AND COMPLEMENTARY DNA

RNA was prepared from thymocytes from the patients and controls with the use of the RNeasy kit

Table 1. Results of Flow-Cytometric Analysis of Patients' Lymphocytes.*

Variable	Patient 1	Patient 2	Patient 3	Control or Normal Range
Markers — % (no./mm ³)				
CD2	22.6 (244)	8.4 (626)	8.0 (190)	75–96
CD3	0.3 (3)	0.1 (7)	0.6 (6)	60–85
CD4	0	0	0	30–60
CD8	0	0	0	15–35
T-cell receptor γ/δ	0	0	0	1–5
CD56	36.0 (391)	7.7 (574)	7.9 (185)	5–20
CD20	62.2 (676)	78.7 (5865)	92.6 (1050)	5–20
Response to phytohemagglutinin — cpm $\times 10^{-3}$	1.1	0.3	0.7	90–130
Serum immunoglobulins — g/liter				
IgG	4.7	3.8	2.5	2.3–14.1
IgM	0.4	0.3	0.6	0.0–1.4
IgA	0.3	0.5	0.2	0.0–0.8

* Flow-cytometric analysis of peripheral-blood lymphocytes with antibodies against CD2, CD3, CD4, CD8, T-cell receptor γ/δ , CD20, and CD56 revealed only a few CD3+ mature circulating T cells, which express neither CD4 nor CD8. Similarly, cells expressing T-cell receptor γ/δ could not be detected in peripheral-blood samples from the patients. In contrast, the number of CD20-expressing B cells was either normal or increased. The number of natural killer cells expressing CD56 and CD2 and serum IgG, IgM, and IgA levels were normal for age in all three patients. The normal range for the lymphocyte count is 2500 to 6000 cells per cubic millimeter. The number of natural killer cells was normal in all patients, and the number of B cells was either normal (in Patients 1 and 3) or increased (in Patient 2).

(Qiagen), according to the manufacturer's suggestions. Random, primed first-strand complementary DNA (cDNA) was synthesized from 5 μ g of total RNA with the use of SuperScript II RNase H Reverse Transcriptase (Invitrogen). Genomic DNA was prepared from Epstein-Barr virus-transformed B-cell lines established from cells from both the patients and the controls or from peripheral-blood mononuclear cells obtained from other family members after Ficoll-Hypaque gradient centrifugation with use of the Wizard genomic DNA-purification kit (Promega), according to the manufacturer's suggestions. Then, cDNA was used to amplify the CD3 δ coding sequence in a polymerase chain reaction (PCR) with use of the primers 5'ATCTACTGGATGAGTTCGGCTGGGAG3' and 5'CTGCTTCTAGAAGCCACCAGTCTCAG3'.

To amplify exon 2 of CD3 δ from genomic DNA by PCR, the primer sequences 5'AACTGTGATATTTTCCCTT3' and 5'CAACCCAAAGGGTTCAGGAAGCAC3' were used. The resultant PCR products were resolved on 1 percent agarose gels, and the appropriate bands were removed and purified with use of the Qiaex II agarose-gel extraction kit (Qiagen). The purified products were directly sequenced with use of the Thermo Sequenase radiolabeled terminator cycle-sequencing kit (Amersham).

MICROARRAY ANALYSIS

The labeled probe was prepared as recommended by the manufacturers of the microarray (Affymetrix), and microarray analysis was performed at the Centre for Applied Genomics (Hospital for Sick Children, Toronto). Briefly, 20 μ g of total RNA from patient and control thymocytes was used as a template for the synthesis of double-stranded cDNA. The cDNA was purified and used as a template for in vitro transcription with biotin-labeled nucleotides (Enzo Diagnostics). Labeled cRNA was fragmented and hybridized to Human Genechip microarrays (HG-U95A and HG-U133A, Affymetrix), which can detect 12,000 and 22,400 messenger RNA (mRNA) species, respectively, all representing annotated genes. The microarrays were scanned and the output files inspected for hybridization artifacts. Arrays without substantial artifacts were analyzed with the use of Microarray suite 5.0 software.^{14,15}

The expression value for each gene was determined by calculating the average differences in intensity (perfect-match intensity minus mismatch intensity) of the pairs of probes for each gene and ensuring that the gene was present in the array. The

differences in expression were calculated by comparing the values for the level of expression of genes from the patient divided by that for the controls. The results have been deposited in the Gene Expression Omnibus at <http://www.ncbi.nlm.nih.gov/geo/> (accession number GSE 609).

RESULTS

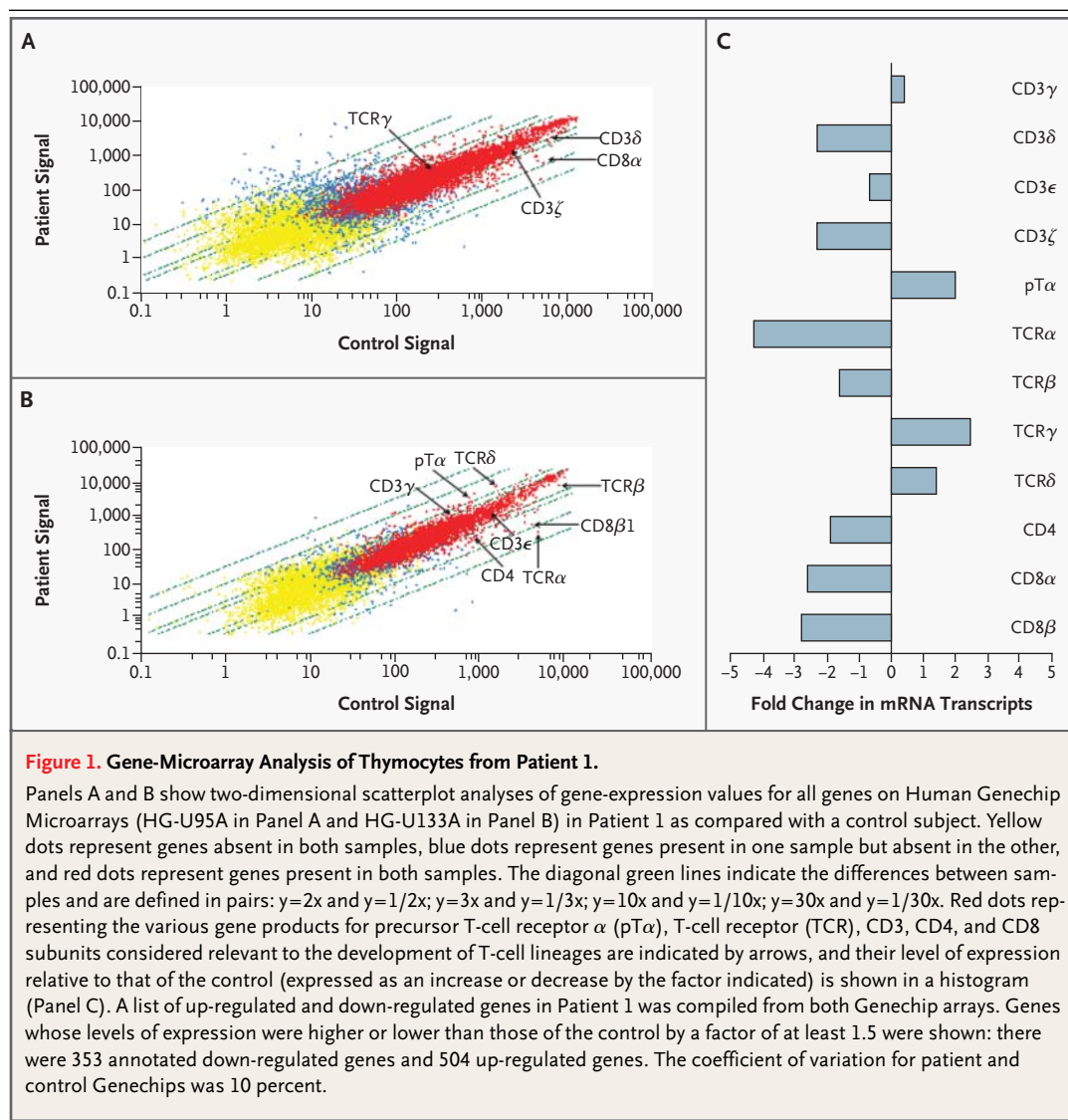
MICROARRAY ANALYSIS OF GENE EXPRESSION IN THE THYMUS OF PATIENT 1

The combination of profound lymphocytopenia and a partially preserved thymus structure suggested that the defect in the three patients with SCID was restricted to T cells and involved a gene controlling T-cell differentiation. To identify the putative genetic defect, we compared gene expression in the thymus of Patient 1 with that of a normal thymus, using oligonucleotide microarrays. Remarkably, only a relatively small number of gene products known to regulate T-cell development were substantially altered in the patient, as compared with the control (Fig. 1A and 1B). Of particular interest were a reduction in T-cell receptor α and T-cell receptor β transcripts by a factor of 4.3 and 1.6, respectively; a reduction in transcripts of CD3 δ and CD3 ζ by a factor of 2.3; and increases in T-cell receptor δ and T-cell receptor γ mRNA by a factor of 1.5 and 2.5, respectively (Fig. 1A, 1B, and 1C).

IDENTIFICATION OF A PREMATURE STOP CODON IN CD3 δ

Despite the lower-than-normal level of CD3 δ and CD3 ζ transcripts in the thymus of Patient 1 (as estimated by microarray analysis), we were able to detect mRNA for both CD3 chains and for CD3 γ and CD3 ϵ using standard reverse-transcriptase PCR (data not shown). Sequence analysis of the CD3 γ , CD3 ϵ , and CD3 ζ cDNA did not demonstrate any abnormalities. However, sequencing of the CD3 δ PCR product from Patient 1 revealed a homozygous C-to-T transition at nucleotide position 202, predicting a premature stop codon, with a truncation at residue 68 (R68stop) in the extracellular domain of the protein. The patient's genomic DNA contained this homozygous mutation in exon 2 of the CD3 δ gene (Fig. 2A, 2B, and 2C).

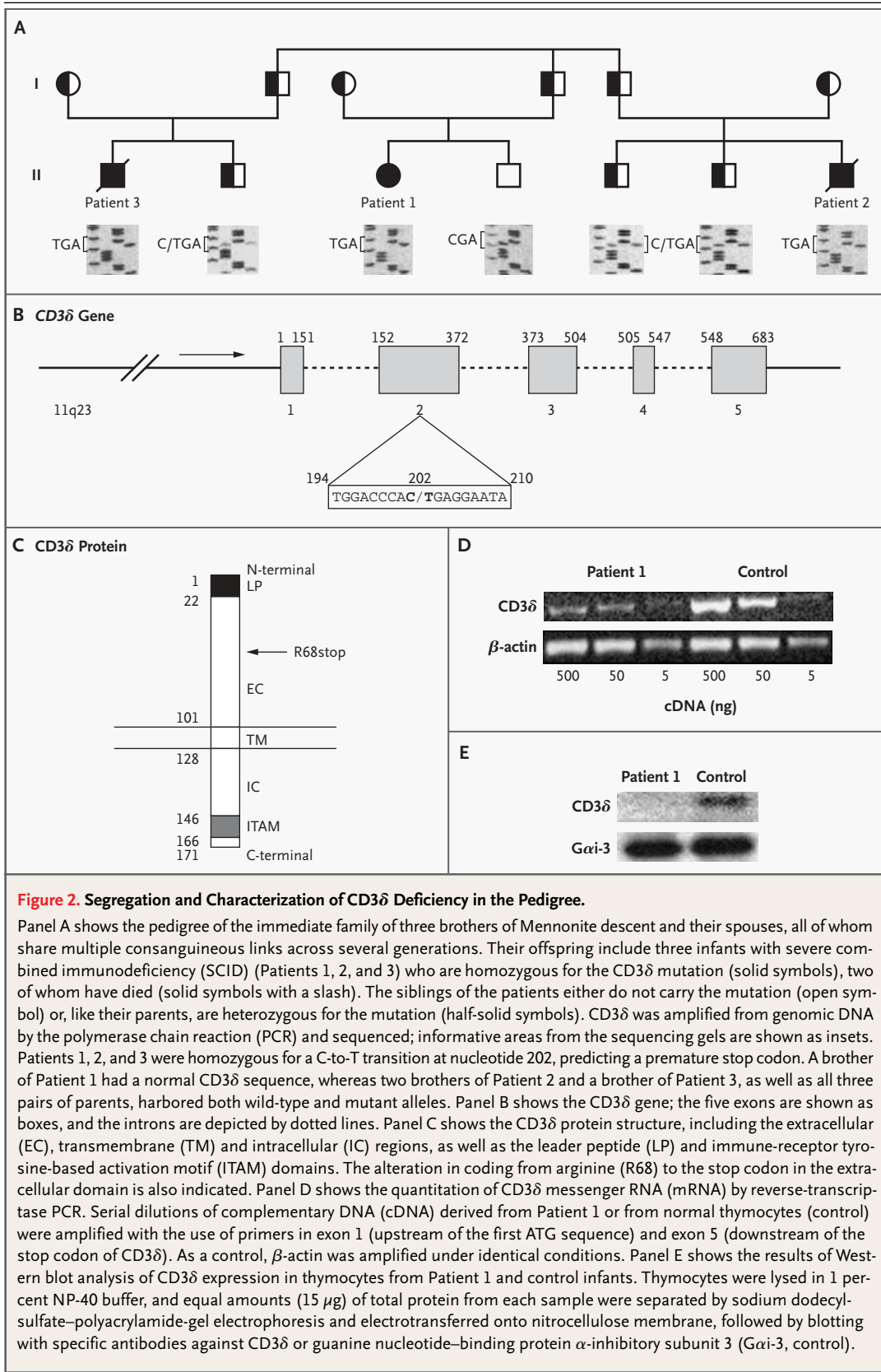
The same homozygous mutation was detected in genomic DNA from the closely related Patients 2 and 3 (Fig. 2A). Both a normal and mutated CD3 δ allele were detected in the genomic DNA sequence of the parents of all three patients, consistent with

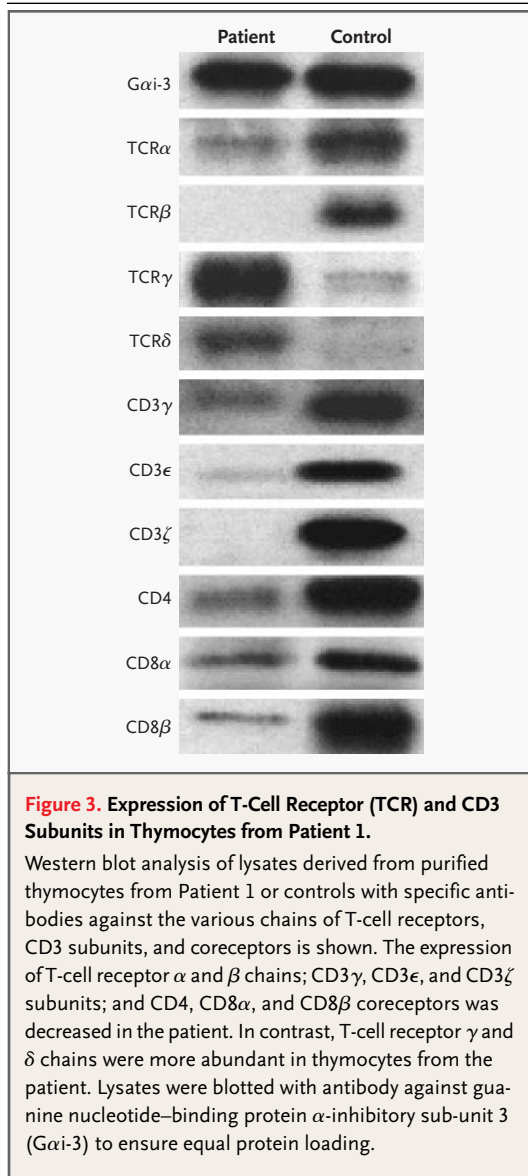


the occurrence of autosomal recessive inheritance. Compatible with this mode of inheritance, siblings of Patients 1, 2, and 3 were either heterozygous for the CD3 δ mutation or completely normal (Fig. 2A). The stop codon within exon 2 can explain the reduction in CD3 δ mRNA (by a factor of 2.3) in the thymus of Patient 1 through a nonsense-mediated decay mechanism.^{16,17} Despite the presence of detectable, albeit reduced, levels of CD3 δ mRNA (Fig. 2D), CD3 δ protein was undetectable by Western blotting (Fig. 2E). The immunodeficiency in these patients thus appears to arise from a heritable mutation of the CD3 δ gene that prevents the synthesis of the CD3 δ protein.

PROTEINS OF THE CD3 COMPLEX IN CD3 δ -/- THYMOCYTES

In comparison with the levels of mRNA for CD3 γ and CD3 ϵ in normal thymocytes, the levels in thymocytes from Patient 1 were marginally altered (Fig. 1C). However, the levels of CD3 γ and CD3 ϵ proteins were lower in the patient's thymocytes than in normal control samples (Fig. 3), possibly because CD3 complexes lacking CD3 δ are rapidly degraded. A similar universal reduction in the expression of CD3 subunits was found in murine CD3 ζ -/- thymocytes.⁷ Unlike the CD3 γ and CD3 ϵ subunits, the CD3 ζ mRNA level was lower (by a factor of 2.3) in the patient's thymocytes than in the control sam-





ples, and levels of the CD3 ζ protein were undetectable on Western blot analysis (Fig. 3).

These CD3 subunits were also assessed immunohistochemically in the thymus tissue of Patient 1 (Fig. 4). Normal thymus showed strong reactivity with antibodies against CD3 γ , CD3 δ , CD3 ϵ , and CD3 ζ . In contrast, CD3 δ , CD3 ϵ , and CD3 ζ were not detected in the patient's thymus. Despite the lack of expression of these CD3 chains, the degree of staining for CD3 γ was similar to that in the control thymus (Fig. 4), suggesting that CD3 γ , even at low cytoplasmic levels, may be transported to the cell membrane independently of the other CD3 chains.

ARREST OF CD3 δ -/- THYMOCYTES AT THE CD4-CD8- STAGE OF DEVELOPMENT

The thymocytes of Patient 1 contained twice as much precursor T-cell receptor α (pT α) gene transcript as did control thymocytes (Fig. 1B and 1C). Since the pT α gene is expressed exclusively by immature thymocytes, these results indicate a block early in the differentiation of T cells in the patient's thymus. Such a block could cause immature CD4-CD8- double-negative thymocytes to accumulate. Indeed, we found reduced levels of CD4, CD8 α , and CD8 β 1 mRNA and protein in CD3 δ -/- thymocytes (Fig. 1 and 3), and immunohistochemical analysis of CD3 δ -/- thymus sections was negative for both CD4 and CD8 (not shown). These results are all consistent with an arrest of differentiation at the CD4-CD8- stage of T-cell development.

THE γ/δ LINEAGE IN CD3 δ DEFICIENCY

The normal thymus contains only a very small number of γ/δ T cells, and these cells constitute up to 5 percent of circulating lymphocytes.¹⁸ The thymocytes from Patient 1 contained increased levels of T-cell receptor γ and T-cell receptor δ transcripts (Fig. 1) and protein (Fig. 3). However, γ/δ T cells could not be detected by flow cytometry in the peripheral blood of the three patients with CD3 δ deficiency (Table 1) or by immunohistochemical analysis of sections obtained from the thymus, lymph nodes, spleen, or gut of Patient 3 (not shown). These results indicate that although the T-cell receptor γ and δ chains are produced, they are not correctly assembled and transported to the cell surface in the absence of CD3 δ .

DISCUSSION

Our three patients with SCID presented with low numbers of circulating T cells and normal numbers of peripheral B cells. This phenotype is typical of SCID caused by mutations in the gene for the common gamma chain (γ c), Janus kinase 3 (*Jak3*), or interleukin-7 receptor α (*IL-7R α*).¹⁹⁻²¹ The γ c and *Jak3* genes are also important in the development of natural killer cells, and the numbers and function of natural killer cells are compromised in many, although not all,^{22,23} such cases of SCID. In SCID arising from aberrations in the *IL-7R α* gene, natural killer cells are preserved.^{24,25} Although our patients had a phenotype in which T cells were absent and B cells and natural killer cells were present, analysis of their γ c, *Jak3*, and *IL-7R α* genes revealed

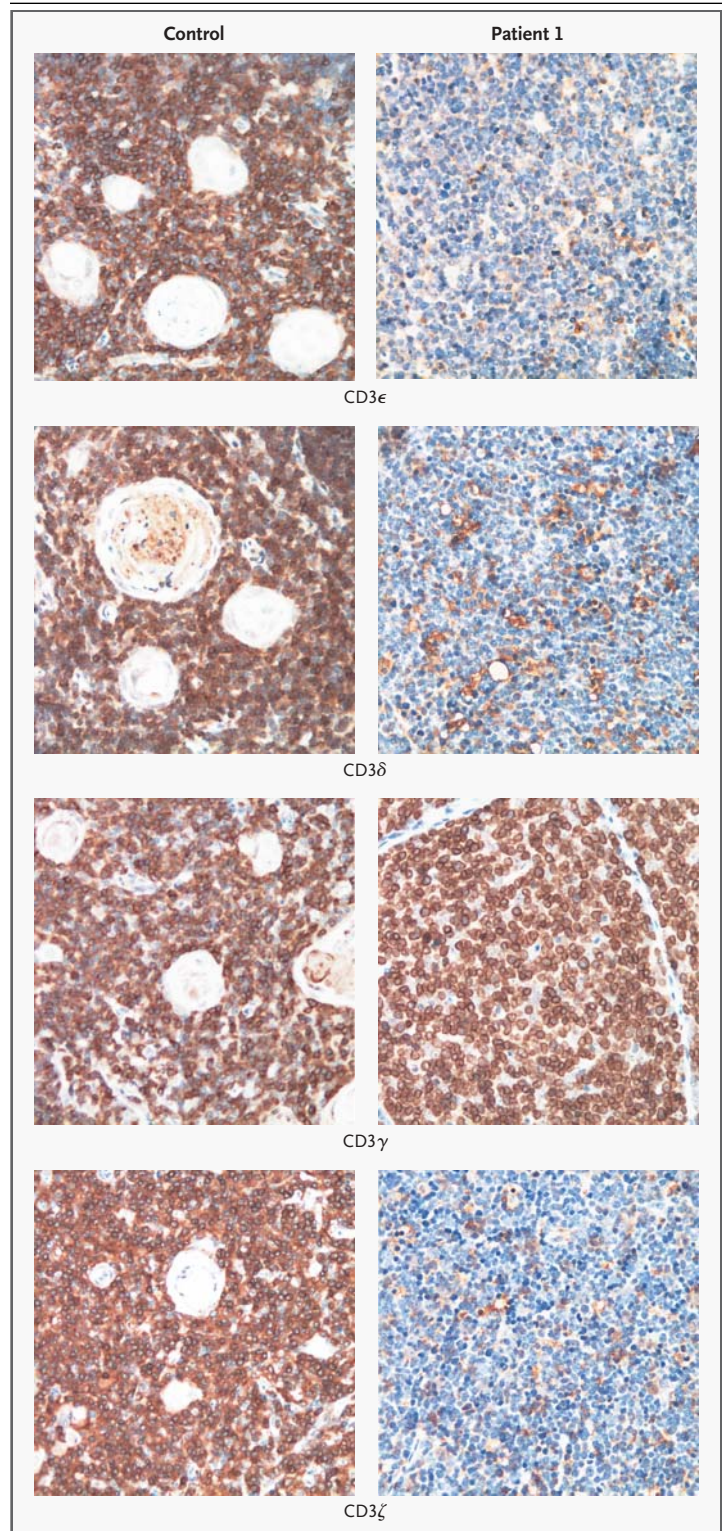
Figure 4. Immunohistochemical Analysis of Thymus Tissue from the Patient and an Age-Matched Control Subject ($\times 20$).

Immunohistologic comparison of thymus sections from Patient 1 with representative thymus sections from an age-matched control subject reveals a complete lack of expression of CD3 ϵ , CD3 δ , and CD3 ζ , but normal expression of CD3 γ , by the patient's thymocytes; sections from the control subject stained strongly for all four CD3 subunits. A distinct demarcation of cortical and medullary structures can be seen in sections from the control but not from the patient. Hassall's corpuscles are also absent in sections from the patient.

no abnormality (data not shown). To define the molecular basis for the immunodeficiency in these patients, we used oligonucleotide microarray analysis of thymocytes isolated from biopsy material as a source of mRNA of T-cell lineage.

This analysis revealed a reduction in mRNA transcripts for all the chains of the CD3 complex except CD3 γ , as well as in mRNA transcripts for the α and β T-cell receptor chains. Extensive biochemical studies have demonstrated that T-cell development is dependent on the function of the CD3 complex,²⁶⁻³⁰ and mice deficient in CD3 γ , CD3 δ , CD3 ϵ , or CD3 ζ have a variable degree of impairment of thymocyte maturation from the CD4 $^-$ CD8 $^-$ to the CD4 $^+$ CD8 $^+$ stage.⁶⁻¹⁰ Our three patients, who had virtually no mature T cells, carried a deleterious mutation in the region of CD3 δ that encodes the extracellular domain of CD3 δ . The mutation, a homozygous C-to-T transition that produced a premature stop codon, resulted in a complete lack of CD3 δ protein in thymocytes. Although only the CD3 δ gene of the CD3 complex was found to harbor a mutation, levels of both the CD3 γ and CD3 ϵ subunits were reduced in CD3 δ $^-$ thymocytes, and CD3 ζ was undetectable by Western blotting. This pattern may reflect the normal content of CD3 subunits in very immature thymocytes.³¹

Precursors of the α/β T-cell lineage undergo three major stages of maturation, defined by the expression of CD4 and CD8. The earliest precursors are designated double-negative, expressing neither CD4 nor CD8. They progress to a stage of dual expression of CD4 and CD8 (double-positive) before committing to the expression of either CD4 or CD8 alone (single-positive) and leaving the thymus. The transition from the double-negative to the double-positive stage requires a productive rearrangement of the T-cell receptor β gene, followed by signaling



through a complex formed by the β chain of the T-cell receptor and the invariant pT α chain.^{1,2} This unit noncovalently associates with the CD3 chains to signal and promote thymocyte differentiation.¹⁻⁵ Subsequently, the T-cell receptor α gene rearranges to allow formation of the mature T-cell-receptor α/β complex.^{1,32}

In our Patient 1, the increased level of pT α transcript and the absence of a T-cell-receptor β gene product in the CD3 δ -/- thymocytes are consistent with the properties of immature T cells that have not rearranged the T-cell receptor α locus and therefore do not display α/β receptors. The marked re-

duction in CD4 and CD8 mRNA and proteins points to a developmental arrest of these immature cells at the double-negative stage of α/β T-cell maturation. Moreover, the lack of detectable γ/δ T cells indicates that the development of this lineage is also arrested in patients with CD3 δ deficiency.

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CORRECTION

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Effect of CD3 δ Deficiency on Maturation of α/β and γ/δ T-Cell Lineages in Severe Combined Immunodeficiency . On page 1822, in the third full paragraph in the right-hand column, the last term listed in line 16 should have been "CD8 β ," not "CD3 δ ," as printed.