

Abbreviations used

BCG:	Bacillus Calmette–Guérin
BSA:	Bovine serum albumin
CMCC:	Chronic mucocutaneous candidiasis
CMV:	Cytomegalovirus
DNA:	Deoxyribonucleic acid
EBV:	Epstein-Barr virus
ELISA:	Enzyme-linked immunosorbent assay
FOXP3:	Forkhead box P3
IFN:	Interferon
IL:	Interleukin
JC:	Jamestown Canyon virus
mRNA:	Messenger RNA
NK:	Natural killer
PAGE:	Polyacrylamide gel electrophoresis
PBL:	Peripheral blood lymphocyte
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate-buffered saline [solution]
PHA:	Phytohemagglutinin
STAT1:	Signal transducer and activator of transcription 1

METHODS**Flow cytometry**

Peripheral blood mononuclear (PMN) cells were obtained by Ficoll-Hypaque density gradient centrifugation, and surface phenotypes were determined by flow cytometry on a Coulter EPICS V flow cytometer (Beckman Coulter, Brea, Calif), with a single argon laser, which analyzes up to 3 colors simultaneously. Single color and/or isotype antibody controls were both used for multicolor stainings. Mature activated T lymphocytes were obtained by culture of PBMC with anti-CD3/CD28 beads (Invitrogen, Life Technologies, Carlsbad, Calif) by following the manufacturer's instructions. Anti-CD45RA-FITC was from Beckman Coulter (Brea, Calif). Anti-CD4-PeCy5(RPA-T4), CD4-FITC(RPA-T4), CD25-PE (BC96), CD31-PE(WM59), FOXP3-PeCy5(PCH101), CD27-PE(LG-7F9), CD27-FITC(LG-7F9), CD19-PeCy5(HIB19), CD95-PE (DX2), Annexin-V-PE were from eBiosciences (San Diego, Calif). A total of 10^5 – 10^6 cells were stained in PBS, 1% BSA, 0.01% sodium azide. Annexin-V-PE binding was assayed in the manufacturer's calcium-containing buffer. In some cases, anti-CD4-PeCy5 was added to the annexin staining to identify viable CD4⁺ T cells.

Serum concentration of immunoglobulin and specific antibodies

Serum concentrations of immunoglobulins were measured by nephelometry. Serum IgE concentration was measured by radioimmunoassay with the IgE PRIST kit (Pharmacia Diagnostics, Dorval, Quebec, Canada). Levels of serum antibodies to tetanus were measured by ELISA, and polio antibody titers were determined by complement fixation.

T- and B-cell proliferative response

Lymphocyte proliferative responses to mitogens, including phytohemagglutinin (PHA) and anti-CD3 antibodies, and to a panel of recall antigens (including *Candida*, tetanus, herpes zoster, and cytomegalovirus [CMV]) were determined by thymidine incorporation.¹⁷ All assays were performed in triplicate and were compared with simultaneously stimulated random normal controls.

Western blotting

Total cell lysates were prepared in RIPA buffer and analyzed by Western blotting after PAGE.¹⁷ Anti-STAT1, anti-pSTAT1 (Y710), anti-Bcl-2, and anti-G protein subunit Gi α were purchased from Santa Cruz Biotechnology Inc (Dallas, Tex). Where necessary, *STAT1* expression levels were quantified by

densitometry scanning and normalized by comparison with matching Gi α levels.

Library preparation, exome capture, sequencing, and data analysis

We followed the user guide for the Ion TargetSeq Exome Enrichment for the Ion Proton System (Life Technologies; User Guide MAN0006730) to build a genomic library by starting from 1 μ g of genomic DNA, except DNA shearing was done to an average of 150-bp by using the adaptive focused acoustics method on a Covaris (Woburn, Mass) S2 instrument. Size selection was performed by using E-gel (Invitrogen) agarose gel electrophoresis. Exome enrichment was performed starting from 500 ng of amplified genomic library. Capture library was diluted to 7 pM and clonally amplified to obtain template-positive ion sphere particles by using the Ion OneTouch 2 System (Life Technologies), and ion sphere particles were sequenced on an Ion Torrent Proton instrument. The sequencing run produced 9.67 Gbp of raw data, with average length of 122 bp. Reads were mapped against the human genome reference hg19 (NCBI build GRCh37), and variant calling was performed by using Ion Torrent Suite default parameters.

Patients

Data were compiled prospectively and retrospectively from medical records and were entered into the Canadian Centre for Primary Immunodeficiency Registry and tissue bank, which has been approved by the SickKids Research Ethics Board (protocol no. 1000005598). This includes consent and assent from patients and parents for genetic analysis, and collection of tissue, including thymus. All the patients were treated primarily at The Hospital for Sick Children.

Patient 1. The patient, a 4-year-old boy, was born to nonconsanguineous parents of English descent. He had a history of oral thrush, which occurred after antibiotic therapy for otitis media (Table 1). He subsequently developed fungal lesions on his face and lips, which responded well to treatment with fluconazole. He had 1 episode of pneumonia and several episodes of otitis media. Growth and development were appropriate for age. He had no family history of severe infections or immunodeficiency. This patient was recently diagnosed, at a relatively early stage of the disease.

Patient 2. A girl born to nonconsanguineous parents of English descent. From the age of 2 years, she had multiple bouts of pneumonia and prolonged episodes of diarrhea, and, since the age of 3 years, she had several bouts of oral thrush after antibiotic therapy. For more critical evaluation, a chest computed tomography was done at 5 years of age, which revealed extensive bronchiectasis in the right middle lobe, left lower lobe, and the lingula. She was found to have generalized lymphadenopathy, an enlarged spleen and liver, and markedly decreased T3 and T4, whereas her thyroid-stimulating hormone level was elevated. By the age of 5 years, she experienced increasing frequency of exacerbation of lung disease as well as chronic diarrhea. At 6.5 years old, she was diagnosed with insulin-dependent diabetes. She died of overwhelming CMV infection at age 7 years (Table 1).

Patient 3. This male patient was born to nonconsanguineous parents of English descent. He had skin eruptions at the age of 6 weeks. From the age of 4 months, he experienced repeated episodes of pneumonia and otitis media, and, at the age of 9 months, had respiratory syncytial virus pneumonitis. At the age of 1 year, he stopped gaining weight and was fed through a nasogastric feeding tube. An impaired ability to make antibodies in response to vaccination was documented at 3 years of age and led to treatment with intravenous immunoglobulin. At age 7 years, he developed hepatitis, probably caused by EBV infection. On examination, he had generalized lymphadenopathy and an enlarged liver and spleen. Acyclovir improved liver function but did not arrest disease progression. He developed pancreatitis at the age of 7 years old and was diagnosed with hypoparathyroidism and Addison disease a year later. He died at the age of 8 years due to overwhelming EBV infection (Table 1).

Patient 4. A female patient was born at term after a normal pregnancy to nonconsanguineous parents of Iranian descent. She had multiple infections, with multiple episodes of pneumonia, including 1 episode due to *Pneumocystis jiroveci* pneumonia at 2 years of age. She had chronic diarrhea

TABLE I. Clinical manifestations

	Patient no.				
	1	2	3	4	5
Infections	Pneumonia, otitis media, oral and skin candidiasis (after antibiotics)	Pneumonias, recurrent diarrhea, oral thrush, systemic CMV, severe chicken pox	Pneumonia, otitis media, EBV	PJP pneumonitis; periorbital cellulitis; severe chicken pox, recurrent zoster, reactivation of BCG; candidiasis of lips, perianal abscess, JC encephalitis	Repeated episodes of pneumonia, neonatal sepsis
Inflammation	None	Chronic diarrhea, chronic lung disease, bronchiectasis, lymphadenopathy, hepatosplenomegaly	Chronic diarrhea, chronic lung disease, bronchiectasis, lymphadenopathy, splenomegaly	Chronic diarrhea, chronic lung disease, bronchiectasis, lymphadenopathy, splenomegaly	Recurrent skin boils, recurrent diarrhea
Endocrinopathy	None	Diabetes mellitus, hypothyroidism	Hypothyroidism, hypoadrenalism	Hypothyroidism	None
Other autoimmune disorders	None	Thrombocytopenia, neutropenia	Chronic hepatitis, neutropenia	Hepatitis, vitiligo	None
Sex	Male	Female	Male	Female	Male
Age/outcome	5 y/alive	7 y/died of CMV disease	10 y/died of liver disease and EBV infection	17 y/died of JC encephalitis	8 y/alive

JC, Jamestown Canyon encephalitis; PJP, *pneumocystis jiroveci* pneumonia.

and subsequently developed recurrent perianal abscesses. At 8 years old, she had a systemic reactivation of BCG and subsequently developed severe herpes zoster. She also had multiple autoimmune manifestations, including hypothyroidism, autoimmune hepatitis, and vitiligo. At the age of 17 years, she was diagnosed with Jamestown Canyon encephalitis, which led to severe brain damage and ultimately her death (Table I).

Patient 5. The patient, an 8-year-old boy, was born at term to nonconsanguineous parent and has repeatedly had oral ulcerations since the age of 13 months. Since the age of 2 years, he also had recurrent skin boils, predominantly on his legs, arms, and buttocks. He also experienced periodic, almost monthly, fevers, which lasted approximately 3 days. He had 3 episodes of lobar pneumonia as well as several episodes of bloody diarrhea. This patient was diagnosed recently and appears to be at an early stage of the disease.

RESULTS

Clinical presentation

We identified a subgroup of patients who presented at our facility with a common pattern of a progressive course of signs and symptoms, including autoimmune manifestations and susceptibility to infections. Most had repeated microbial, viral, or fungal lung infections, which led to progressive and permanent damage to the lungs (Table I). Three of the 5 patients eventually died of overwhelming viral infections, including CMV, EBV, or Jamestown Canyon virus. Three patients had oral thrush, albeit not a prominent manifestation, which frequently occurred after antibiotic or corticosteroid treatment and was readily responsive to topical treatment. Uniquely, all these patients also were characterized by a progressive loss of T-cell and natural killer (NK) cell numbers with age. Ultimately, their immune system declined to include a humoral defect with an inability to produce specific antibodies as well as profound T-cell dysfunction, suggestive of combined immunodeficiency.¹⁶

All the patients had various degrees of enlarged lymph nodes as well as hepatosplenomegaly. The array of autoimmune features was wide, including hypothyroidism, diabetes mellitus, hepatitis,

vitiligo, and others (Table I). The multiple endocrinopathy suggested a possibility of CMCC among other possibilities. Nevertheless, none of these patients demonstrated the typical disease profile for CMCC. Although they did manifest increased susceptibility to infections, candidiasis did not play a dominating role as previously reported for homozygous mutations in *AIRE* or heterozygous dominant gain of function *STAT1* mutations.¹² Moreover, with the exception of BCG reactivation in 1 patient, the patients did not experience mycobacterial infections, which have been associated with autosomal recessive loss of function mutations in *STAT1*. This unique presentation prompted an attempt to study the underlying defect by analyzing candidate genes combined with whole exome DNA sequencing.

Genetic analysis

Whole exome sequencing combined with analysis of candidate genes converged in the identification of mutations in *STAT1*. An exome library of patient no.1 was sequenced to an average read depth of 121X, with 80.7% of the reads mapped to the reference genome, and 61.0% of these reads being on target to the capture baits. Approximately 93.8% of the targeted bases were sequenced to at least 20X. A total of 27,166 single nucleotide variants and 10,175 indels were called. Variants supported by fewer than 10 reads were discarded. Filters were applied to the remainder to remove synonymous and noncoding variants, followed by retention of variants with polyphen scores of ≥ 0.5 and sorting intolerant from tolerant scores SIFT (Sorting Intolerant From Tolerant scores) of < 0.01 , which have a significant predictive impact of the substitution on protein structure and function. We identified 45 potentially damaging variants. Among these, a G>A substitution (c1154T) in *STAT1*, which causes an amino acid threonine to methionine (T385M) substitution ($P = 7.94 \times 10^{-5}$) was identified as possibly being responsible for

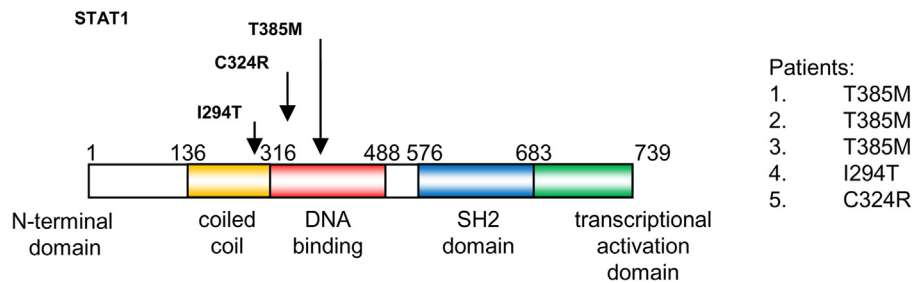


FIG 1. *STAT1* cartoon. The major domains of *STAT1* are labeled with boundary amino acids identified. Two of the 3 disease-associated mutations lie within the DNA binding domain, whereas the third is adjacent to them at the C-terminal end of the coiled coil domain.

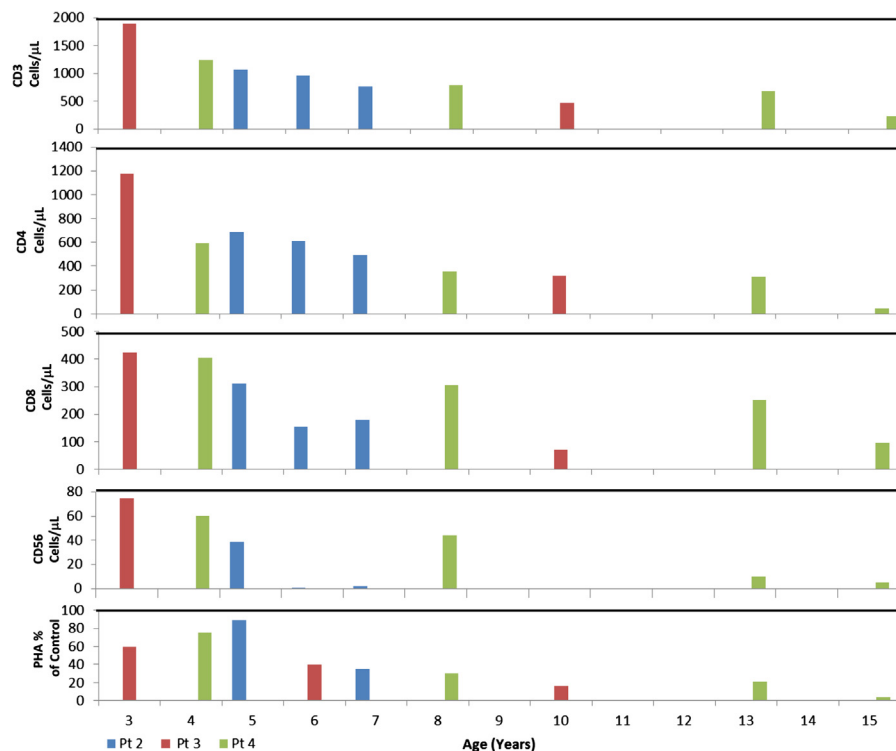


FIG 2. Progressive decrease in T- and NK-cell numbers with age. T-lymphocyte subset and NK-cell numbers in peripheral blood were assessed over time by flow cytometry. Proliferative responses to PHA mitogen were performed in parallel.

the observed genotype. This substitution was validated by Sanger sequencing.

The above change was identified as the most common mutation in this group of patients (Fig 1). Patient no. 1 was heterozygous for this mutation, retaining 1 normal wild-type allele. The same T385M mutation was identified posthumously in a further two of the patients (patient nos. 2 and 3). Once again, T385M was present in the heterozygous state. In all 3 cases, the T385M mutations were determined to be *de novo* and not present in either parent. This appeared consistent with the poor outcome of the disease, with patient nos. 2 and 3 dying at 7 and 8 years of age, respectively, which suggests that T385M was unlikely to undergo inheritance. The further 2 patients in our group who displayed progressive T-cell decline were also found to possess *STAT1* mutations. Patient no. 4 was posthumously identified with a *de novo* I294T *STAT1* mutation, whereas patient no. 5 was found to possess a heterozygous

C324R *STAT1* mutation, which, once again, was not present in either parent.

Immune evaluation

Consistently in these patients, the number of circulating T lymphocytes declined over time, with the first signs being detectable by 3-5 years of age. In patient nos. 2, 3, and 4, a long follow-up allowed for complete documentation of this decline (Fig 2). In patient nos. 1 and 5, who had been diagnosed more recently, this phenomenon could not yet be fully appreciated, although we already observed the apparent early stages of this decline.

Thymus biopsy specimens were obtained early in the disease, at ages 2.5 years and 1.5 years in patient nos. 2 and 3, respectively. Hematoxylin-eosin staining of thymus sections obtained from patient no. 2 (Fig 3) showed a clear corticomedullary definition,

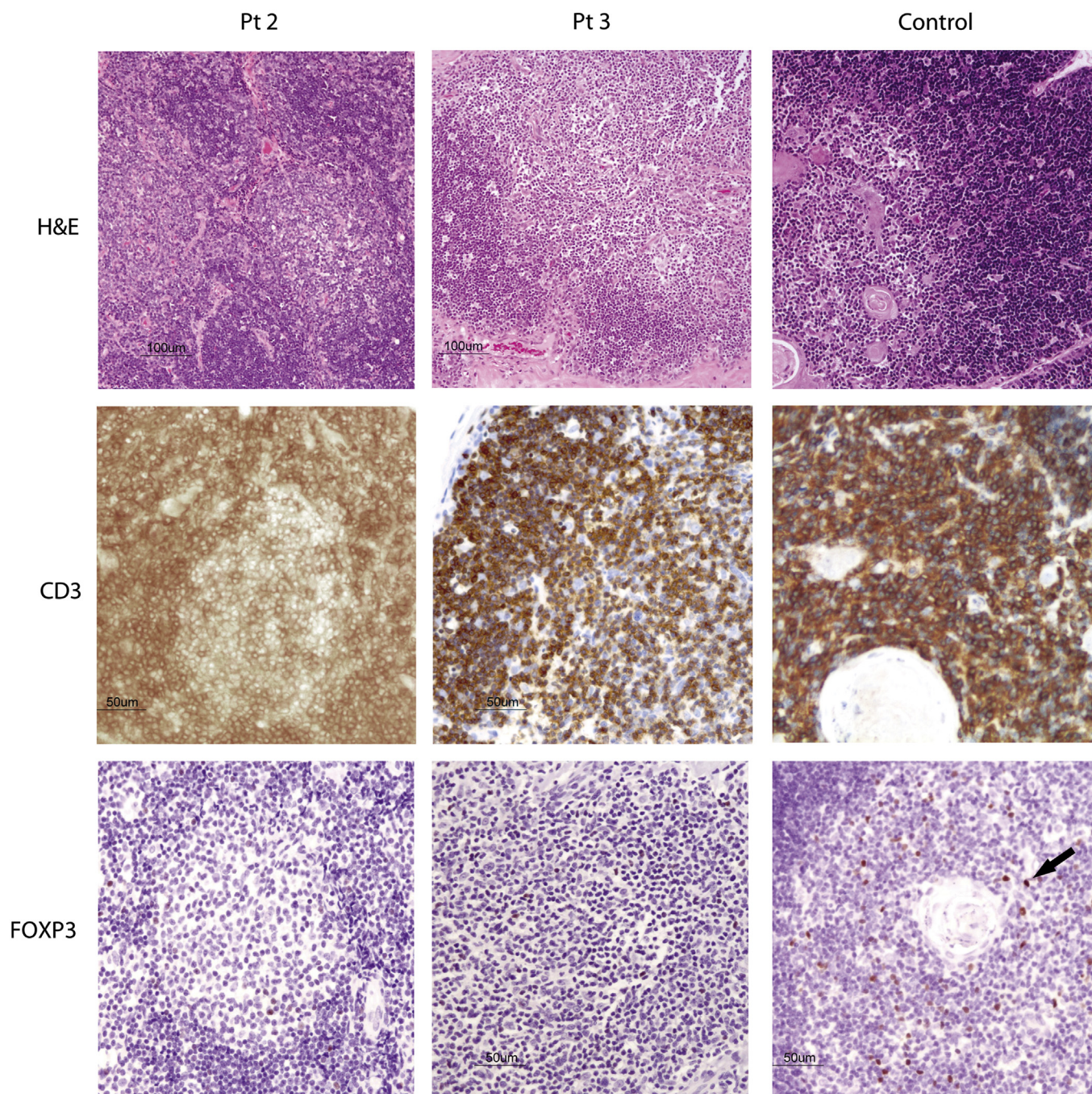


FIG 3. A lack of Hassall corpuscles and reduced expression of FOXP3. Representative sections of thymus from patient nos. 2 and 3 (*STAT1* T385M^{+/-}) were stained with H&E or antibodies to CD3 ϵ or FOXP3 as indicated. Control thymus tissue was obtained during a cardiac surgical procedure of an individual who was immunologically normal.

with CD3⁺ thymocytes present, which demonstrated both CD4⁺ and CD8⁺ staining (not shown) but an absence of Hassall corpuscles. For patient no. 3, similar observations were made. The thymus demonstrated normal lobular architecture, with individual lobules that showed clear division into cortex and medulla but with no identifiable Hassall corpuscles, a pattern typical of patients who present with combined immunodeficiency.

Both patients showed a slight cortical hypocellularity compared with controls, and immunohistochemistry revealed a

reduction in staining with anticytokeratin, which indicates decreased epithelial elements, consistent with the lack of Hassall corpuscles. Staining with antibodies to the forkhead box P3 (FOXP3) transcription factor revealed an absence of FOXP3⁺ regulatory T cells in either patient, despite their clear presence in control thymus, which suggests that maturation of this lineage was defective (Fig 3). This could be consistent with the increasing incidence of autoimmune phenomena observed with age in these patients.

TABLE II. Immune evaluation

	Patient no.					Normal range
	1	2	3	4	5	
Markers (cells/ μ L)						
CD3	1328	765	356	228	850	1200-2600*
CD4	617	495	235	45	534	650-1500*
CD8	420	180	65	96	243	370-1100*
CD20/19	256	126	421	0	446	270-870*
CD56	220	2	0	5	35	100-480*
Mitogen responses, patient/control†						
PHA‡	570/1510	67/187	62/153	27/680	822/2315	>50%
α CD3	0.1/68	43/233	36/130	2/104	206/455	>50%
Antigen responses‡						
Candida	<20	<20	ND	<20	ND	>20
CMV	<20	<20	ND	<20	ND	>20
Zoster	<20	<20	ND	<20	ND	>20
Tetanus	<20	<20	ND	<20	ND	>20
Immunoglobulin (g/L)						
IgG	6.0	13.5	8.4	21.3	3.6	6.7-17.3
IgM	0.2	1.5	0.9	0.20	<0.02	0.5-3.1
IgA	0.5	3.9	1.5	0.70	0.3	0.4-3.7
IgE	ND	<3.5 μ mL	ND	ND	ND	<5 μ mL
Specific antibodies						
Tetanus (IU/mL)	0.09	<0.01	<0.01	<0.01	0.28	>0.05
Mumps	(-)	ND	ND	(-)	(-)	>1:16
Measles	(-)	ND	ND	(-)	(-)	>1:16
Rubella	(+)	ND	ND	(-)	(-)	>1:16
Isohemagglutinin	1:8/1:2	<1:8	ND	<1:8	1:4/1:4	>1:8
Polio 1,2,3		<1:8	<1:8	<1:8		>1:16
Thymus biopsy	ND	Dysplastic thymus	Dysplastic thymus	ND	ND	ND

ND, Not done; (-), undetectable; (+), more than 1:16.

*See Ref 31.

†Expressed as stimulation index.

‡*In vitro* proliferation of T cells in response to antigens.

In vitro mitogenic responses of peripheral blood T cells from patients to PHA or anti-CD3 were universally reduced, and responses to the antigens *Candida*, tetanus toxoid, herpes zoster, and CMV were markedly decreased (Table II). Abnormal responses were evident before the decline in lymphocyte numbers, in which assessment was possible in patient nos. 1 and 3 (Fig 2, Table II).

Analysis of peripheral T cells from patient nos. 1 and 5, who appear to be in an early stage of immune deterioration, revealed a reduction in CD3⁺ T-cell numbers compared with the normal range in patient no. 5 only (Table II). No significant abnormality in the presence of thymic naive (CD4⁺, CD31⁺, CD45RA⁺) T cells was detected in either patient, in agreement with T cell receptor excision circles analysis, which also suggested that thymic output levels were still within normal variation (not shown). Effector memory T cells (CD4⁺, CD27⁻, CD45RA⁻) were similarly normal. An increased percentage of central memory cells (CD4⁺CD27⁺CD45RA⁻: 63% of all CD4⁺ T cells versus 54% in typical control) were observed in patient no. 1 but not in patient no. 5. Similar to the *STAT1* knockout mouse,¹⁸ both patients demonstrated a small decrease in the percentage of circulating CD4⁺CD25⁺ T cells, with fewer FOXP3⁺ T cells (patient 1 = 10.4/ μ L; control range, 23-54 cells/ μ L) (Fig 4, A), potentially a forerunner of the absence of regulatory T-cell lymphocytes observed in thymus biopsy specimens taken from patient nos. 2 and 3 (Fig 3).

In parallel with the decline in T-cell numbers, a gradual decrease in the number of circulating NK cells also was observed in most patients (Fig 2). Although certain immunoglobulin levels were consistently normal in all but 1 patient, the ability to respond to vaccination or infections by production of specific antibodies was notably impaired (Table II). Although absolute numbers of CD19⁺ B lymphocytes were normal in both of the younger patients (nos. 1 and 5), analysis of B-cell subsets revealed a significant reduction in the percentage of CD27⁺ memory B cells (P1 = 17/ μ L, P2 = 35/ μ L, normal controls 80-435 cells/ μ L) (Fig 4, B), which provides a possible reason for the universal failure to produce specific antibodies in all patients.

***In vitro* cytokine production**

In vitro cytokine production assays revealed a sharp reduction in IL-2 secretion by PBL from both patient no. 1 (Fig 4, C) and patient no. 5 (not shown) in response to stimulation with PHA or anti-CD3, possibly explaining the reduced T-cell proliferation in these patients (Table II). Addition of exogenous IL-2 significantly rescued proliferation (not shown), which suggests that defective IL-2 secretion was primarily responsible for this failure to respond. Surprisingly, T cells of patient no. 1 also failed to secrete IFN- γ (Fig 4, C). In contrast, phorbol 12-myristate 13-acetate/ionomycin or IL-12 induced IFN- γ secretion responses were normal. IL-17 secretion was absent in response

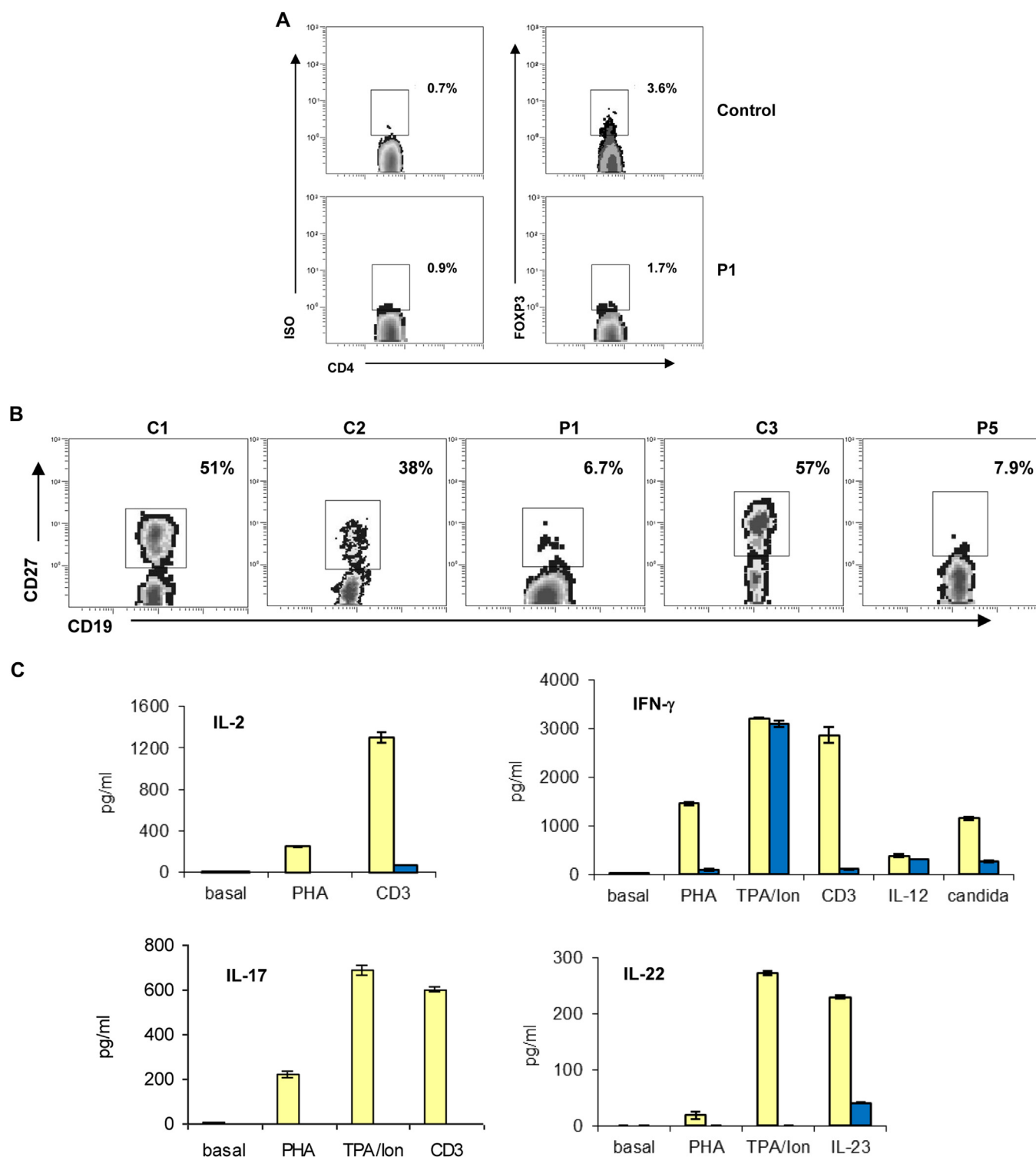


FIG 4. A, Decreased FOXP3⁺ CD4 T cells in patient no. 1 (P1). B, Decreased CD19⁺CD27⁺ memory B cells in patient nos. 1 and 5 (P5). Normal controls (C1, C2, C3). C, Decreased cytokine secretion. P1 and control PBMC (1×10^6) were stimulated as indicated and culture medium collected after 48 hours. Levels of indicated cytokines were determined by ELISA of triplicate samples. Yellow = control; blue = P1.

to all stimuli examined, which suggests that IL-17 secreting Th17 cells were likely absent. Similarly, IL-22 production in response to 12-O-tetradecanoylphorbol-13-acetate/ionomycin was also absent (Fig 4, C).

STAT1 function and expression

All the *STAT1* mutations found in our patient group (T385M, T294I, C384R) lie within or near the DNA binding domain of the transcription factor, although not directly at the DNA:protein

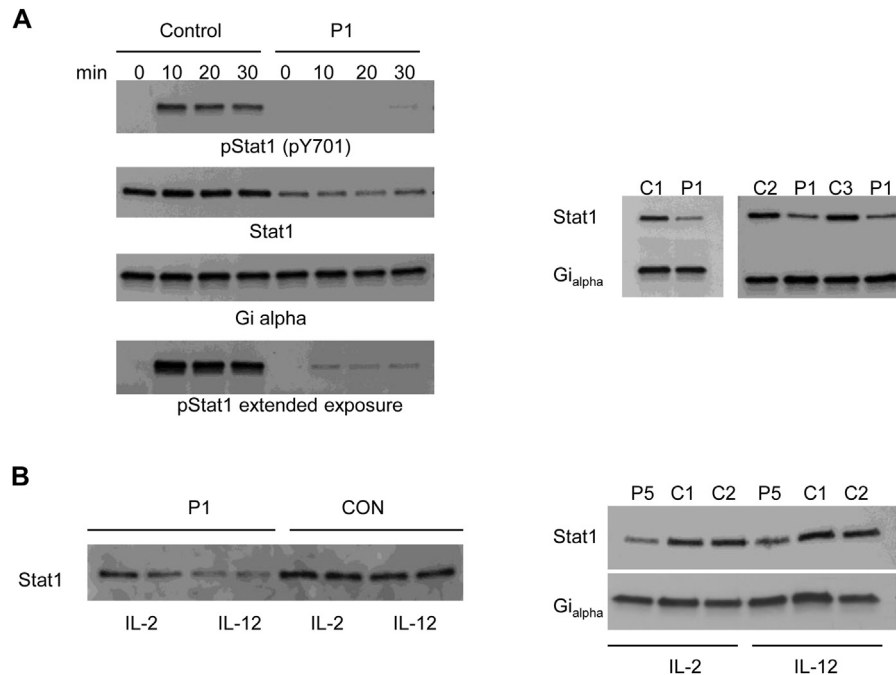


FIG 5. Decreased *STAT1* activation and expression. **A, left panel,** *STAT1* activation (anti-pY701) and expression (anti-*STAT1*) in IFN- γ stimulated patient no. 1 (P1) and control PBMC cell lysates. Anti-Gi α loading control; **right panel,** *STAT1* expression in 2 independent samples of P1 PBMC compared with 3 normal random controls (C1, C2, C3). **B, Patient no. 1 (left panel), patient 5 (P5) (right panel) and control (CON, C1, C2) PBMC** were cultured with anti-CD3/CD28 plus IL-2, with or without Th1 promoting IL-12. Whole-cell lysates were blotted for Stat1 and Gi α . All experiments were repeated at least twice with independent samples. Controls were randomly selected healthy adults.

interface (Fig 1). Alterations within this domain have been shown to effect DNA binding, potentially as gain of function mutations, which demonstrated both increased activating phosphorylation (for unclear reasons) and DNA binding,¹³ similar to mutations in the coil-coil domain.¹² However, contrary to observations in patients with gain of function *STAT1* mutations, PHA and anti-CD3 stimulation in our cohort failed to elicit production of IL-2 and IFN- γ (Fig 4, C), which suggested a possible loss of *STAT1* activity.

Consistent with reduced function, IFN- γ stimulation of PBMC (T385M^{+/-}) of patient no. 1 showed a depressed level of *STAT1* activation by pY701 *STAT1* Western blot analysis (Fig 5, A). Moreover, subsequent anti-*STAT1* blotting revealed that patient no. 1 expressed less than 50% of the level of *STAT1* protein found in controls (relative levels varied from 20%-40% with different controls). This reduction was evident both when comparing Ficoll-purified PBMCs and purified mature T lymphocytes. Furthermore, patient no. 5 (C324R^{+/-}) demonstrated the same decrease in *STAT1* expression ($\leq 50\%$) (Fig 5, B). Based on these findings, we were forced to conclude that, in primary cells, both T385A and C324R *STAT1* were either not translated, or were unstable, possibly due to insufficiently stable protein folding.

PBMCs from patient nos. 2 and 3, also T385M^{+/-}, or from patient no. 4 (I294T^{+/-}) were not available for study; however, given the similarity in clinical presentation, we hypothesize that I294T *STAT1* protein expression may be similarly affected. In summary, primary cells heterozygous for either T385M or C324R *STAT1* demonstrated decreased levels of *STAT1* expression and depressed *STAT1* responses, which suggest that

the functional consequences of these heterozygous mutations have more in common with a knockout than the gain of function phenotype.

Elevated Fas receptor expression

One possible explanation for the low and potentially decreasing number of T cells in these patients could lie in increased susceptibility to the induction of apoptosis. Increased levels of Fas receptor (CD95) expression have been reported to mark deterioration of the immune system in the elderly.¹⁹⁻²³ Upon examination, patient nos. 1 and 5 both displayed significantly higher levels of CD95 expression on T cells, even when compared with much older controls (≥ 60 years old) (Fig 6, A and B). As expected, the CD4⁺ CD45RO⁺ patient and control memory T cells were CD95⁺, but levels of cell-surface CD95 expression on individual cells were elevated in both patients. Furthermore, a percentage of CD45RA⁺ naive CD4⁺ T cells, which were negative for CD95 in controls, displayed CD95 expression in both patient nos. 1 and 5, similar to observations in aging immune systems.²³

Increased Fas expression is not always associated with increased T-cell apoptosis, presumably due to an absence of matching changes in the expression of pro- and antiapoptotic proteins.^{19,21} Levels of antiapoptotic Bcl-2 were found to be normal in T cells of both patients; however, staining for apoptosis (annexin-V-PE) of T cells that were activated with anti-CD3/anti-CD28 beads (Invitrogen) and then expanding in the presence of IL-2 revealed significantly increased levels of apoptosis in patient no. 5 compared with controls (Fig 6, C). Similar findings were observed with

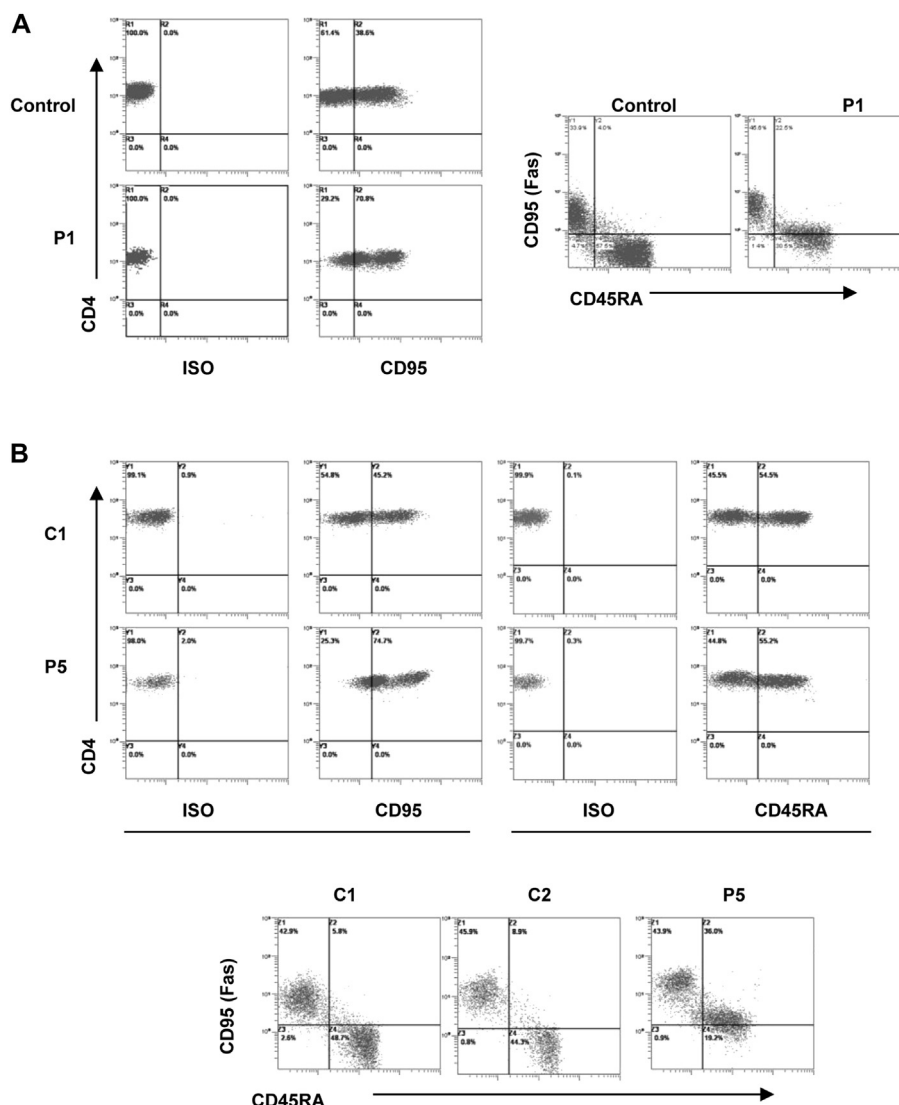


FIG 6. Increased Fas expression. Freshly isolated PBMC cells from patient no. 1 (**A**) (P1), and patient 5 (**B**) (P5), and controls (C1, C2), were stained as indicated and analyzed by flow cytometry. **C**, Representative annexin-V-PE staining of samples of control (CON) and patient no. 5 (P5) T cells growing in culture with anti-CD3/CD28 and IL-2. Control is randomly selected healthy adult. Total annexin-V binding and CD4⁺ cell specific binding are shown.

patient no. 1 (not shown). Thus, in these patients with $STAT1^{+/-}$, increased T-cell apoptosis seems likely to contribute to the decline in T-cell numbers with time.

DISCUSSION

The characterizing features of the patients presented here with heterozygous *STAT1* mutations are life-threatening severe infections that increase with age, sometimes into their second decade of life, accompanied by variable autoimmune features. These manifestations appear related to gradually declining B- and T-cell functions, with an increasing physical loss of these lymphocyte populations. Even in the early stages, when T-lymphocyte numbers are still within normal range, T-cell function is significantly impaired as observed by the inability of T385A^{+/−} and C284R^{+/−} T cells to produce IL-2 after stimulation.

Common to T385M^{+/−} and C284R^{+/−} T cells, expression of Fas death receptor was significantly increased over normal and

increased susceptibility to Fas activation induced cell apoptosis may contribute to the progressive decline in T-cell numbers. Thymus biopsy specimens of 2 patients (with T385M^{+/−}, I294T^{+/−}) also demonstrated architecture consistent with defects in the continuing generation of naive T cells. The development of autoimmune phenomena was noted in several patients, possibly related to the decline in regulatory T-cell numbers and the disruption of T-cell homeostasis, which potentially allows expansion of self-reactive cells. In all cases, the heterozygous *STAT1* mutations in these patients were not present in either parent, which suggests *de novo* generation. This would be entirely consistent with the apparently fatal nature of these particular *STAT1* mutations due to an increasing inability to combat infection.

The T385M mutation of *STAT1* has been reported as a gain of function mutation, which demonstrates increased activation in an EBV B-cell line.¹³ We also examined *STAT1* activation in an

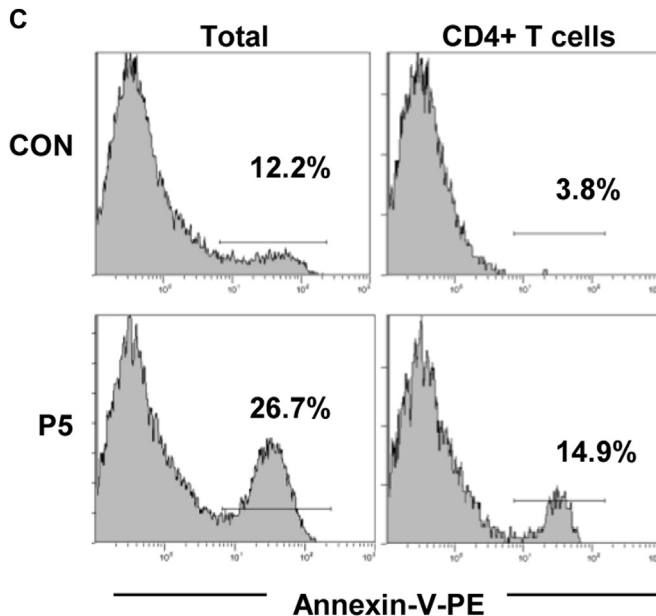


FIG 6. (Continued)

EBV-transformed B-cell line created from the PBL of patient no. 1 and came to a similar conclusion. However, although results of these studies suggested that even minimal amounts of T385M *STAT1* should be highly active, there was no evidence of such activity in the primary cells of patient no. 1, the most likely explanation being that the protein had been degraded after synthesis. Therefore, the gain of function observed upon forced overexpression would appear to be largely physiologically irrelevant. We speculate that the anomalous expression observed in the EBV B cells may occur as the result of the specific relationship between EBV and *STAT1*, whereby *STAT1* is directly upregulated and activated by EBV gene products^{24,25} and is necessary for the virus to maintain the nonlytic phase (as it exists in EBV-transformed B-cell lines),²⁶ and the high levels of heat shock protein expression encountered in EBV-transformed lines,²⁷⁻²⁹ which may help stabilize the mutant *STAT1* protein.

In addition to T-cell decline, a notable loss of NK cells with age was also seen in our patients. *STAT1*-deficient mice have been shown to possess normal numbers of NK cells, albeit with impaired NK cytolytic activity.³⁰ Peripheral expansion in response to infection is impaired in these animals due to the failure to respond to IFN- γ ; however, the developmental process appears normal.

Our patients clearly differ significantly from those with *STAT1* mutations, predisposing to a susceptibility to mycobacterial disease, for example, heterozygous Q463H or L706S,⁶ or solely autosomal dominant CMCC, for example, heterozygous R274W or C174R.^{12,15} There are several other known mutations of *STAT1* that significantly reduce expression; however, these all also manifest differently. They include homozygous P696S, which disrupts mRNA splicing and results in expression of the mutant protein at 10% of normal *STAT1* levels.⁷ P696S is accompanied by severe but treatable intracellular bacterial and viral infections. Homozygous K201N results in a 70% reduction in *STAT1* expression, also due to an RNA splicing error.¹¹ Patients with K201N (and K211R) demonstrate mycobacterial fungal and

infection, often severe. In contrast, disseminated BCG and potentially lethal viral infection at an early age are hallmarks of a complete lack of *STAT1* expression that results from homozygous deletion of 2 bp, insertion of 1 bp (both causing a translational frame shift), or L600P.^{8,10}

Numerous heterozygous *STAT1* mutations have now been reported that do not appear to be associated with a progressive T or NK cell decline as observed in our patients. We hypothesize that only the subset with decreased, but not absent, *STAT1* protein expression as a result of heterozygous mutation manifests this phenomenon. It is surprising that a 50% reduction in the absolute *STAT1* level should have such dramatic repercussions, particularly when the remnant protein would be predicted to be wild type in such a model. We, therefore, speculate that (a) degradation of mutant *STAT1* protein is accompanied by “bystander degradation” of wild-type *STAT1* due to the formation of mutant wild-type heterodimers after synthesis and resulting in a more than 50% reduction in expression; and (b) that the mutant *STAT1* protein pool is not degraded in its entirety and that the remnant protein may still act in some dominant negative fashion with regard to T-cell function and/or survival.

Analysis of the accumulating evidence suggests prognosis for patients with decreased T-cell *STAT1* expression is poor. Two of 3 patients with heterozygous T385M *STAT1* succumbed to infection, whereas the third patient appears to be in the early stages of T-cell loss. The literature includes a single report of 2 patients with heterozygous T385M *STAT1*, 1 patient, a 12-year-old boy (presenting at 2 years old with severe recurrent oral thrush and diagnosed with CMCC), and a second boy who died at 14 years old of intravascular coagulation and pulmonary insufficiency of unknown etiology.¹³ Faced with the apparently fatal nature of this condition, it is possible that such patients may be candidates for restorative bone marrow transplantation. However, the influence of potentially low *STAT1* expression in tissues, which supports hematopoietic cell development remains to be seen. We, therefore, recommend that patients with recurrent infections, with or without persistent oral thrush and declining numbers of circulating lymphocytes, should be tested for a possible mutation in *STAT1*. If indeed *STAT1* is mutated, then close attention should be paid to repeated infections and autoimmune manifestations.

In conclusion, we have shown an association between heterozygous *STAT1* mutations that cause a reduction in *STAT1* expression and a predisposition to a progressive severe combined immunodeficiency due to a time-dependent decline in T- and B-lymphocyte numbers and function. This unique combination of progressive combined immunodeficiency with a fatal outcome at a young age, associated with heterozygous *STAT1* mutation represents a distinct disease entity.

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Clinical implications: Patients with recurrent infections and autoimmunity, with or without typical features of chronic mucocutaneous candidiasis, should be tested for *STAT1* mutations as these patients are at high risk of fatal infection.

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