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GATA-2 *anomaly and clinical phenotype of a sporadic case of lymphedema, dendritic cell, monocyte, B- and NK-cell (DCML) deficiency, and myelodysplasia*

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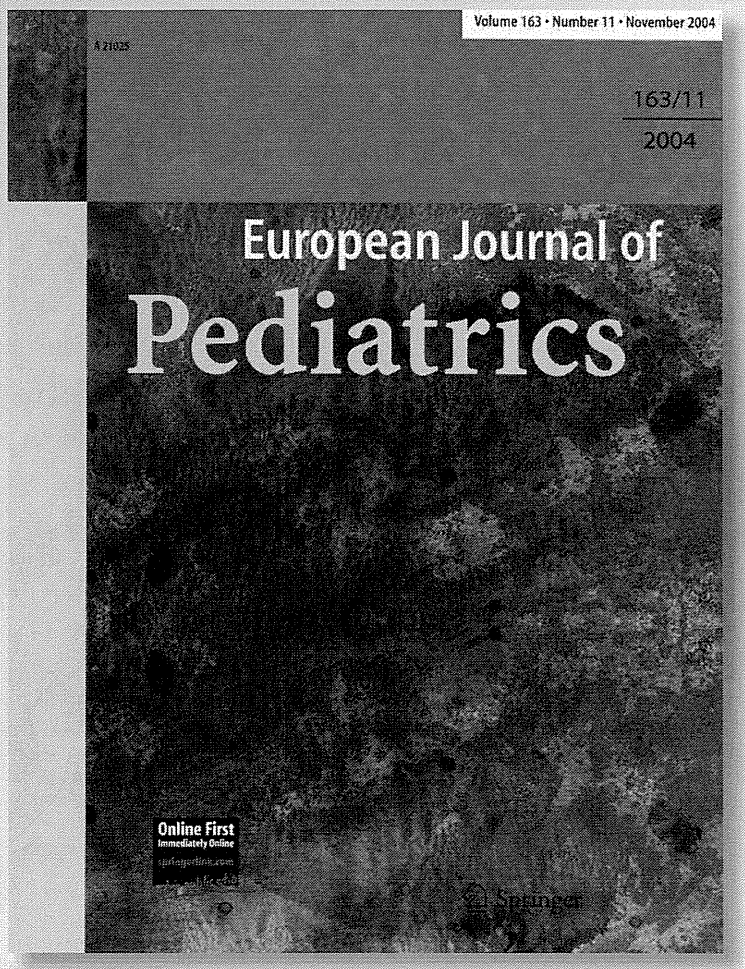

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CASE REPORT

GATA-2 anomaly and clinical phenotype of a sporadic case of lymphedema, dendritic cell, monocyte, B- and NK-cell (DCML) deficiency, and myelodysplasia

Hiroyuki Ishida · Kosuke Imai · Kenichi Honma · Shin-ichi Tamura · Toshihiko Imamura · Masafumi Ito · Shigeaki Nonoyama

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Abstract A Japanese patient presented with lymphedema, severe *Varicella zoster*, and *Salmonella* infection, recurrent respiratory infections, panniculitis, monocytopenia, B- and NK-cell lymphopenia, and myelodysplasia. The phenotype was a mixture of the monocytopenia and mycobacterial infection (MonoMAC) and Emberger syndromes. Sequencing of the *GATA-2* cDNA revealed the heterozygous missense mutation 1187 G>A. This mutation resulted in the amino acid mutation Arg396Gln in the zinc fingers-2 domain, which is predicted to cause significant structural change and prevent a critical interaction with DNA. Functional analysis of the patient's *GATA-2* mutation is required to understand the relationship between these distinctive syndromes.

Keywords Emberger syndrome · MonoMAC · Monocytopenia · B- and NK-cell lymphopenia · Immunodeficiency · Myelodysplasia

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Recent studies have characterized a novel primary immunodeficiency known as monocytopenia and mycobacterial infection (MonoMAC), also known as dendritic cell, monocyte, B and NK lymphoid (DCML) deficiency. This form of immunodeficiency occurs either as an autosomal dominant form or sporadically. It is primarily characterized by persistent and profound peripheral monocytopenia, diagnostic B- and NK-cell lymphocytopenia, and variable T cell lymphocytopenia, along with increased susceptibility to mycobacterium or papilloma virus infections [1, 2, 13]. Moreover, most patients with MonoMAC eventually develop acute myelogenous leukemia (AML) following myelodysplastic syndrome (MDS). Another rare disorder called Emberger syndrome (MIM614038) is characterized by congenital deafness and primary lymphedema of the lateral lower limb; typically, onset occurs in childhood and is associated with a predisposition to MDS or AML in addition to other minor anomalies such as hypotelorism and long tapering fingers. It is also a sporadic or familial disorder [8]. Familial MDS/AML without other hematopoietic defects has also been reported [6]. Surprisingly, it was reported recently that these three distinctive syndromes are all caused by *GATA-2*

mutations, which suggests that these syndromes are different phenotypes caused by the same genetic alteration [5–7, 9]. Here, we report the case of a patient with a *GATA-2* mutation bearing the characteristic features of MonoMAC/Emberger syndrome.

Case report

The patient was the second child of non-consanguineous parents. Neither the parents nor the elder brother had a history of increased susceptibility to infection. The medical history of the patient included BCG vaccination 3 months after birth without any side effects and a severe *Varicella zoster* infection at 2 years of age. After that, she suffered repeated upper and lower respiratory tract infections that required antibiotics. At 4 years of age, the patient's peripheral blood showed mild neutropenia and profound monocytopenia ($0–20\times10^6$/L), and mild hypocellularity but no dysplasia was observed in the bone marrow. At 8 years of age, she experienced a prolonged *Salmonella* enterocolitis infection. Lymphedema in the left leg first appeared at 13 years of age. She subsequently developed recurrent panniculitis. Recently, the patient (now 19 years old) was admitted to hospital with fever (with no apparent cause) and panniculitis (Fig. 1a). She had mild hypotelorism and lymphedema, with warts on her left leg (Fig. 1b). Her mental ability was appropriate for her age. An immunodeficiency was first suspected after the severe *Varicella zoster* and *Salmonella* infections during early childhood. The most recent recurrent episode of fever supported this suspicion.

Peripheral blood analysis revealed a white blood cell count of 1.5×10^9/L with 45% neutrophils, 54 % lymphocytes, and 1 % monocytes, a hemoglobin level of 11.0 g/dl, and a platelet count of 146×10^9/L. Flow cytometric analysis of the peripheral blood also revealed a deficiency in dendritic cells (lineage$^-$/DR$^+$/CD123$^+$ or CD11c$^+$ cells, 0%), B cells (CD19$^+$ cells, 0.7%), and NK cells (CD3$^-$/CD56$^+$ cells, 0.5%), and profound monocytopenia (CD14$^+$ cells, 0.2%). Lymphocytes comprised 97% T cells (CD4/8 ratio, 0.54), 33% of which were TCR $\gamma\delta^+$ T cells. Immunological analyses revealed IgG, IgA, IgM, and IgE levels of 711, 65, 131,

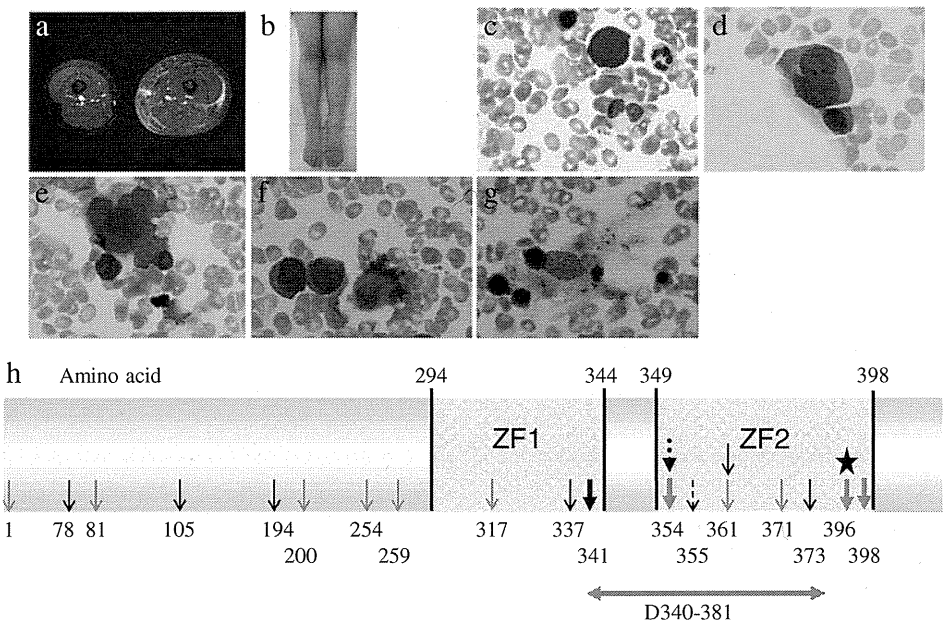

Fig. 1 Clinical and bone marrow features and *GATA-2* protein mutation sites. **a** A gadolinium-enhanced T2-weighted MRI image of the left thigh was performed when the patient developed panniculitis at 19 years of age. An increased signal was observed in the subcutaneous tissue and fascial layers. **b** After she was cured, the patient showed lymphedema in her left leg. **c–g** Bone marrow taken at the same time revealed decreased granule numbers within neutrophils and a pseudo-Pelger anomaly (**c**), binucleation (**d**), and megaloblastic changes in erythroblasts, dysplastic nuclei in megakaryocytes (**e**) and micromegakaryocytes (**f**), and hemophagocytosis (**g**). **h** Depiction of the *GATA-2* protein mutations previously identified in MonoMAC/DCML deficiency and Emberger syndrome. ZF1 and ZF2 are functional DNA-binding domains. The *star* indicates the Arg396Gln mutation identified in the present case. *Arrows* indicate previously reported mutations. These include missense, nonsense, and frameshift mutations (*short downward arrows*, respectively) and long deletions (*horizontal arrows*). *Black arrows* denote mutations associated with Emberger syndrome, *gray arrows* denote mutations associated with MonoMAC syndrome/DCML deficiency, *long horizontal arrows* indicate long deletions that have been observed in MonoMAC syndrome/DCML deficiency, *broken black arrows* denote mutations associated with familial MDS/AML, and *bold arrows* denote multiple pedigrees with the same mutation

and 5 mg/dl, respectively, and lymphocyte stimulation responses to phytohemagglutinin at the lower limits of the normal range. Antibody memory responses to infections contracted in early childhood (*Varicella* and *measles*) were maintained, and fibroblast sensitivity to radiation was normal. Flow FISH analysis of peripheral blood lymphocytes revealed normal telomere length; however, the peripheral blood contained 160 copies/μg WT1-mRNA (the upper limit of normal is 50 copies/μg RNA), and bone marrow aspirates showed hypocellularity, particularly of myeloid and lymphoid cells. Strikingly, despite monocytopenia in the peripheral blood, $CD64^+$ macrophages (accompanied by a few hemophagocytes) were observed in bone marrow specimens. Significant trilineage dysplasia was also present (Fig. 1c–g). Cytogenetic and chromosomal breakage analyses showed normal results. Meanwhile, profiles of familial peripheral blood showed a white blood cell count of 5.6×10^9/L with 50% neutrophils, 30% lymphocytes, and 8% monocytes, a hemoglobin level of 15.1 g/dl, and a platelet count of 199×10^9/L in the father; 5.1×10^9/L with 51 % neutrophils, 36% lymphocytes, and 9% monocytes, 10.7 g/dl, and 225×10^9/L in the mother; and 6.6×10^9/L with 41% neutrophils, 45% lymphocytes, and 10% monocytes, 15.4 g/dl, and 208×10^9/L in the brother. Flow cytometric analysis of peripheral blood samples taken from these family members showed a normal frequency of B cells ($CD19^+$ cells) and NK cells ($CD3^-/CD56^+$ cells) (the father 11 and 8%, the mother 10 and 12%, and the brother 9 and 15%, respectively). Taken together, these findings suggested that the patient might have sporadic MonoMAC/Emberger syndrome.

Sequencing of *GATA-2* cDNA revealed a 1187 G>A heterozygous missense mutation. This mutation resulted in an Arg396Gln substitution in the zinc finger-2 domain, which is predicted to cause significant structural changes that prevent critical interactions with DNA (Fig. 1h). Furthermore, sequencing of cDNA from her healthy familial members revealed no mutations, including 1187 G>A in *GATA-2* gene. Ultimately, the patient was diagnosed with MonoMAC/Emberger syndrome with a de novo *GATA-2* mutation.

Discussion

GATA-2 plays a critical role in both hematopoietic stem cell development and the maintenance of normal adult stem cell homeostasis [10]. It is likely that the significant protein structural alterations caused by mutations in *GATA-2* result in loss-of-function or have a dominant-negative effect on the DNA-binding ability of wild-type *GATA-2* [9] It seems reasonable to suggest that the loss of hematopoiesis-indispensable transcription factor activity results in impaired hematopoietic-cell differentiation and hematopoietic stem cell exhaustion; this in turn may promote the development of related diseases such as MDS and AML. Additional genetic alterations may also be required.

The patient's phenotype included hypotelorism, primary lymphedema (which had an onset during childhood before the recurrent episodes of panniculitis), peripheral monocytopenia, B- and NK-cell lymphocytopenia, neutropenia since early childhood, and myelodysplasia. The Arg396Glu mutation in *GATA-2* identified in this patient was not detected in 150 healthy individuals [7]. Taken together, these factors confirmed the diagnosis of MonoMAC/Emberger syndrome with a de novo *GATA-2* mutation; however, the *GATA-2* mutations alone cannot explain the phenotypic diversity between these three syndromes (MonoMAC, Emberger syndrome, and familial MDS/AML) and the presented patient. Interestingly, she developed neither BCG dissemination nor severe lymphadenitis after her BCG

Table 1 Summary of the clinical features of MonoMAC, Emberger syndrome, and the present case

	MonoMAC/DCML deficiency	Emberger syndrome	Present case
DCML[a] deficiency	+	+/−	+
MDS/AML	+	+	+
Lymphedema	ND	+	+
Deafness	ND	+/−	−
Hypotelorism	ND	+/−	+
Long slender fingers	ND	+/−	−
Mycobacterial infection	+	ND	−
Fungal infection	+/−	+/−	−
Papillomaviral infection/warts	+	+	+
Severe *varicella* and/or *Salmonella* infection	+/−	ND	+
Pulmonary alveolar proteinosis	+/−	ND	−
Panniculitis/erythema nodusa	+/−	+/−	+

ND not described, *MDS* myelodysplastic, *AML* acute myelogenous syndrome, + most cases, +/− some cases

[a]Dendritic cell, monocyte, B and NK lymphoid syndrome

vaccination 3 months after birth. This indicates normal functioning of tissue macrophages, because protective immunity to mycobacteria is dependent upon the interleukin (IL)-12/IL-23-interferon (IFN)-γ axis, possibly mediated by intracellular killing of phagocytes following the production of IFN-γ by CD4 T lymphocytes in response to IL-12/IL-23 secreted by infected macrophages [3]. Patients with MonoMAC/DCML deficiency show very low numbers of circulating monocytes and no detectable myeloid or plasmacytoid dendritic cells in the peripheral blood, but relatively normal numbers of Langerhans cells and tissue macrophages accompanied by prominent hemophagocytosis in the bone marrow [1, 2]. This supports the idea that tissue and marrow macrophages, in addition to Langerhans cells, may be maintained by a distinct precursor from circulating monocytes or dendritic cells [1].

Mansour et al. [8] reported that the age of onset of MDS/AML in Emberger syndrome is 9–14 (median 11) years of age. This appears to be earlier than that of MDS/AML in MonoMAC syndrome (7–52 years, median 32 years) [2]. Moreover, the level of WT1-mRNA in the peripheral blood increases significantly as MDS progresses and is a strong predictor of rapid AML transformation in adult patients with de novo MDS [11]. The level of WT1-mRNA in the peripheral blood of the current patient was as high as that in patients that show worse survival than those with a low level WT1 mRNA (10^2–10^4 vs. $<10^2$ copies/μg) [12]. However, it is unclear whether phenotypic variation and increased WT1 mRNA level are related to hematological disease progression. In any case, neutropenic patients who suffer recurrent infections and/or MDS are likely to need a transplant in the near future. Therefore, for such cases, we perform hematopoietic stem cell transplantation with a reduced intensity conditioning regimen before the disease has progressed [4]. Table 1 summarizes the clinical features of MonoMAC, Emberger syndrome, and the present case.

Our observations suggest that children with recurrent or prolonged common infections that respond to antibiotics and recover well may suffer from unknown primary immunodeficiencies. Although the relationship between *GATA-2* and lymphedema or deafness requires further investigation, tissue-specific lesions such as lymphedema provide important clues to primary immunodeficiencies that also affect non-hematopoietic cells.

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Conflict of Interest Statement The authors declare no competing financial interests.

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Multiple Reversions of an IL2RG Mutation Restore T cell Function in an X-linked Severe Combined Immunodeficiency Patient

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Abstract Reversion mosaicism is increasingly being reported in primary immunodeficiency diseases, but there have been few cases with clinically improved immune function. Here, a case is reported of X-linked severe combined immunodeficiency (SCID-X1) with multiple somatic reversions in T cells, which restored sufficient cell-mediated immunity to overcome viral infection. Lineage-specific analysis revealed multiple reversions in T cell receptor (TCR) αβ+ and TCRγδ+ T cells. Diversity of the TCRVβ repertoire was comparable to normal and, furthermore, mitogen-induced

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proliferation of the patient's T cells was minimally impaired compared to healthy controls. *In vivo* and *in vitro* varicella antigen-specific T cell responses were comparable to those of healthy controls, although a reduced level of T cell receptor excision circles suggested that recent thymic output was low. During long-term evaluation of the patient's immunologic status, both the number of CD4+ and CD8+ T cells and T cell proliferation responses were stable and the patient remained healthy. This case demonstrates that multiple but restricted somatic reversions in T cell progenitors can improve the clinical phenotype of SCID-X1.

Keywords Severe combined immunodeficiency · reversion · multiple

Introduction

X-linked severe combined immunodeficiency (SCID-X1) is a recessive hereditary disease characterized by a lack of T cells and natural killer (NK) cells. Without stem cell transplantation, persistent infections with opportunistic organisms uniformly lead to death in the first 2 years of life, except in those with atypically attenuated phenotypes [1–3]. Recently, spontaneous genetic reversion has been reported in primary immunodeficiency disorders. Somatic reversion mosaicism is considered to be 'natural gene therapy'; however, few cases are reported with reversions that restore functional immunity [4–9]. Here, an atypical case of SCID-X1 with somatic mosaicism due to multiple reversions in T cells, which restored sufficient T cell immunity, is described.

Materials and Methods

Patient

A male infant was born prematurely at 34 weeks and 4 days of gestation with a birth weight of 1,660 g to healthy parents. There was no family history of consanguinity or immunodeficiency. He was well until 14 months of age, when he started to have recurrent bacterial respiratory tract infections. At the age of 21 months, laboratory tests were performed. Patient results were compared to age-matched normal controls (controls). Examination of serum Ig revealed a decreased level of IgG (IgG, 1.93 g/L [range of controls: 7.15–9.07 g/L]), and normal levels of IgA (IgA, 0.33 g/L [range of controls: 0.22–1.44 g/L]) and IgM (IgM, 0.72 g/L [range of controls: 0.34–1.28 g/L]). His serum IgG was constantly under 2.0 g/L. In addition, he had a reduced number of CD4+ cells (358/μl, [mean of controls: 1,683± 874]) and CD56+ cells (39/μl [mean of controls: 306± 207]), while CD3+ cells (1,803/μl [mean of controls: 2,997±1,751]), CD8+ cells (1,067/μl [mean of controls: 1,683±874]) and CD19+ cells (1,850/μl [mean of controls: 1,114±976]) were within the normal limits. The patient's T cell proliferative response to phytohemagglutinin (PHA) (stimulation index (S.I.) of 172 [range of controls: 105–225]) and to concanavalin-A (Con-A) (S.I. of 140 [range of controls: 68–154]) was within the normal ranges for his age. From these data, he was diagnosed with common variable immunodeficiency (CVID) at that time. Intravenous immunoglobulin therapy was started and he remained in good health thereafter. Without receiving vaccination, varicella infection at 5 years of age did not cause fever, and he was successfully treated with oral acyclovir at an outpatient clinic. At 9 years of age, warts developed and spread over his body, and he was referred to our hospital for assessment of his immunological status. Physical examination revealed neither detectable lymph nodes nor tonsils, and his thymus appeared hypoplastic on CT scan. Before the laboratory studies were performed, informed consent was obtained from the patient and his parents, in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

Flow Cytometry

Flow cytometric analysis was performed according to standard protocols with a FACSCalibur flow cytometer (Becton Dickinson, USA). The following fluorochrome-conjugated antibodies (Abs) were used for flow cytometric analysis: CD3 (clone SK7), CD4 (clone CK3), CD8 (clone SK1), CD14 (clone M5E2), CD19 (clone SJ25C1), CD56 (clone B159), CD45RA (clone HI100), CD45RO (clone UCHL1) (BD Biosciences Pharmingen, USA), TCRαβ (clone IP26A), TCRγδ (clone IMMU 510) (Beckman Coulter, Inc., USA), CCR7 (clone 150503, R&D Systems Inc., USA), CD27 (clone O323, eBioscience, Inc., USA), CD132 (clone TUGh4, BD Biosciences Pharmingen), and rabbit anti-Human IgD polyclonal Ab (DAKO Japan Co., Japan).

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Sequencing of Genomic DNA and cDNA, and Subcloning Analysis

Peripheral blood mononuclear cells (PBMCs) were obtained from the patient and his parents and various cell lineages were sorted using a FACSVantage (Becton Dickinson). The genomic DNA was isolated from the sorted samples and the cDNA was obtained using reverse transcriptase Super Script II (Invitrogen, USA) with Oligo $(dT)_{20}$ primer. Genomic DNA and cDNA were amplified with the proofreading PCR enzyme, KOD –Plus– (Toyobo, Japan). Direct sequencing analysis of all exons of the *IL2RG* gene, including introns at least 50 bases adjacent, were performed on an ABI 3700 (Applied Biosystems, USA). For analysing revertant subclones in each PBMC lineage, the genomic DNA and the cDNA isolated from sorted cell fractions were amplified by PCR with primer pairs 5′-TCCCAGAGGTT CAGTGTTTTG-3′ and 5′-TTGCAACTGACAGCCA GAAG-3′, and 5′-CGCCATGTTGAAGCCATC-3′ and 5′-TTGCAACTGACAGCCAGAAG-3′, for the region spanning exons 2 and 3 of *IL2RG,* respectively. These PCR products were subcloned using a TOPO TA Cloning Kit (Invitrogen) and sequenced.

T cell Functional Assays

To obtain PHA-induced T cell blasts, PBMCs were stimulated with PHA (Invitrogen) at 1:100 dilution and cultured in RPMI 1640 (RPMI) supplemented with 5 % fetal calf serum (FCS) with recombinant human IL-2 (50 IU/ml, kindly provided by Takeda Pharmaceutical Company, Japan) at 37 °C for 7 days. After being rested in RPMI with 5 % FCS overnight, the T cell blasts were stimulated with various concentrations of IL-2 for 48 h, and [^{3}H]-thymidine uptake assays were performed as previously described [8]. T cell receptor (TCR) Vβ repertoire analysis and CDR3 spectratyping were performed as described [10, 11]. *In vitro* cytokine production against varicella zoster virus (VZV) antigen was performed as previously described [12]. Spots were enumerated automatically using the KS ELISPOT system (Carl Zeiss). The *in vivo* delayed-type hypersensitivity (DTH) reaction to subcutaneous purified VZV antigen (BIKEN, Japan) was performed as previously described [13]. The T cell receptor excision circles (TRECs) from the patient PBMCs were measured as previously described [14].

Tyrosine Phosphorylation of STAT5 by IL-2

PBMCs (1×10^6) were cultured in RPMI with 5 % FCS at 37 °C for 2 h and then treated with or without IL-2 (10,000 U/ml) for 10 min. The cells were fixed and permeabilized with BD Cytofix Buffer and Phosflow Perm Buffer III (BD Biosciences Pharmingen) according to the manufacturer's instructions. After washing with PBS containing 1 % FCS, the cells were stained with mouse anti-pSTAT5 (pY694) (clone 47, BD biosciences), anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry.

Results and Discussion

At the age of 9 years, the patient presented with generalized warts and no detectable lymph nodes and tonsils. This, coupled with his prior hypogammaglobulinemia, prompted a re-evaluation of his immunological status. He showed a decreased level of IgA and a normal level of IgM but no isohemagglutinin. Mitogen-induced proliferation assays showed a slightly reduced response to PHA and Con A (Table I). Surface marker analysis of PBMCs revealed slightly decreased levels of CD3+ and CD4+ T cells, and a normal level of CD8+ T cells (Table II). Naïve CD4+ T cells

Table I Laboratory investigations (patient aged 9 years)

		Patient (IVIG)	Healthy controls
Blood counts			
White blood cells	(count/μl)	7,400	3,600–9,800
Neutrophil	(count/μl)	4,773	3,000–5,000
Lymphocyte	(count/μl)	2,028	2,500–4,500
Monocyte	(count/μl)	340	200–950
Eosinophil	(count/μl)	252	0–700
Basophil	(count/μl)	7	0–150
Red blood cells	($\times10^6$ count/μl)	5.15	4.08–5.07
Hemoglobin	(g/dl)	12.5	11.6–14.1
Platelet	($\times10^3$ count/μl)	275	201–409
Serum Immunoglobulin levels			
IgG	(g/L)	7.69	10.79±2.63
IgA	(g/L)	0.26	2.46±0.91
IgM	(g/L)	1.08	0.83±0.21
IgD	(mg/L)	<6	55±16
IgE	(IU/mL)	<5	<170
isohemagluttinin		Undetectable	
T cell proliferation			
None	(cpm)	163	127–456
Phytohemagglutinin	(cpm)	16,800	20,500–56,800
Concanavalin A	(cpm)	16,600	20,300–65,700
DTH reaction to subcutaneous varicella virus antigen			
Erythematous change (mm in diameter)		18	≧5.0

Control values of blood counts are shown as the range from 95 % of healthy children aged 9 to 12 years. Control values of serum immunoglobulin levels are based on children aged 8 to 10 years and are shown as the mean ± SD. IVIG indicates monthly intravenous infusion of 2.5 g immunoglobulin

Table II Surface marker analysis of peripheral blood mononuclear cells (patient aged 9 years)

	Patient (count/μl)	Healthy controls (count/μl)
CD3+	1,080	2,813±1,197
CD4+	357	1,699±850
CD8+	582	972±457
TCRαβ+	890	2,154±1,004
TCRγδ+	190	324±182
CD4+CD45RA+CCR7+	8	1,290±756
CD8+CD45RA+CCR7+	25	655±503
CD8+CD45RA+CCR7-	114	221±95.3
CD8+CD45RA-CCR7+	33	30.1±27.6
CD8+CD45RA-CCR7-	410	132±87.4
CD19+	894	1,238±605
CD19+CD27+smIgD-	0.4	86.6±61.3
CD19+CD27+smIgD+	14.3	172±123
CD3-CD56+	Undetectable	271±186

Absolute numbers of cells expressing surface markers are shown. Healthy control values are from children aged 2 to 9 years and are shown as mean ± SD

(CD4+/CD45RA+/CCR7+), naïve CD8+ T cells (CD8+/CD45RA+/CCR7+), and both switched memory B cells (CD19+/CD27+/smIgD-) and unswitched memory B cells (CD19+/CD27+/cmIgD-) were scarce. In addition, natural killer (NK) cells (CD3-/CD56+) were absent. This suggested the existence of a genetic defect causing lack of NK cells, such as an *IL2RG* deficiency and *JAK3* deficiency, and therefore the expression of IL2RG (also known as the common gamma chain or CD132) was examined by flow cytometry. Reduced expression was found on B cells and monocytes, although T cells expressed normal levels of CD132 (Fig. 1a). To determine whether CD132-dependent signal transduction was functioning, STAT5 phosphorylation was analyzed on patient CD4+ and CD8+ T cells in response to IL-2. It was found to be comparable with that of normal controls (Fig. 1b). In addition, a proliferation assay of PHA-induced T cell blasts in response to exogenous IL-2 was performed (Fig. 1c). This confirmed that the patient T cells, which were expressing normal levels of CD132, also had intact IL-2 signaling.

To elucidate the genetic cause of the lineage-dependent CD132 expression abnormalities, *IL2RG* genomic sequencing was performed in various cell lineages. Genomic sequencing of *IL2RG* in B cells, monocytes and buccal mucosa revealed a point mutation, c.284-15A>G, in intron 2 of *IL2RG*. This has been reported as a causative mutation of SCID-X1 [15], producing aberrant mRNA with an insertion of 14 bases spanning nucleotide −14 to −1 of exon 3 (Fig. 2a, b). Genomic sequencing of *IL2RG* in T cells showed overlapping bases at and around the mutation sites, while the cDNA of *IL2RG* from

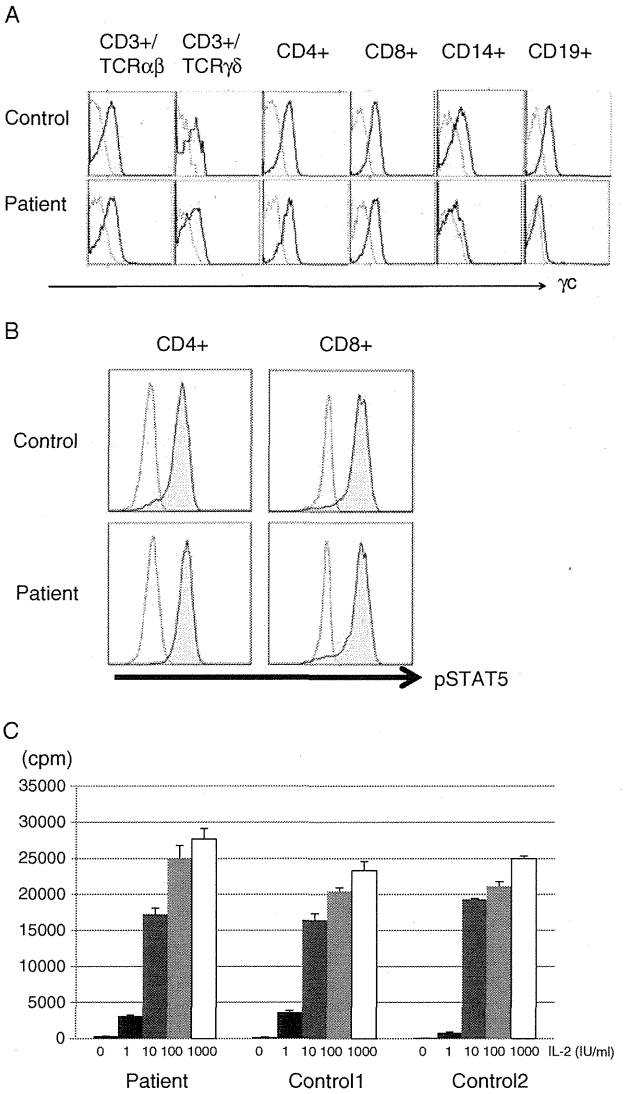

Fig. 1 IL2RG expression and T cell function at 9 years old. **a** Surface expression of IL2RG on PBMCs from the patient and healthy control gated according to the expression of the indicated lineage surface markers. *Black lines* indicate staining for IL2RG (with anti-CD132 Ab) and *gray lines* indicate staining with the isotype control. Data represent one of three independent experiments. **b** STAT5 tyrosine phosphorylation in patient and control CD4+ and CD8+ cells after incubation with (*shaded histograms*) or without IL-2 (*open histograms*). **c** Proliferation of PHA-induced T cell blasts in response to IL-2 stimulation from the patient and two controls. Data are shown as means ± SD

the T cells was normal (Fig. 2a, b). Genomic sequencing of PBMCs from the patient's mother confirmed her as a carrier of the mutation. The possibility of maternal engraftment was excluded by FISH analysis of sex chromosomes (data not shown), and it was concluded that the patient inherited the mutation from his mother and that reversion occurred in the patient's T cells, which led to somatic mosaicism.

To explore the reversions that could have occurred to restore normal *IL2RG* expression in the patient's T cells,

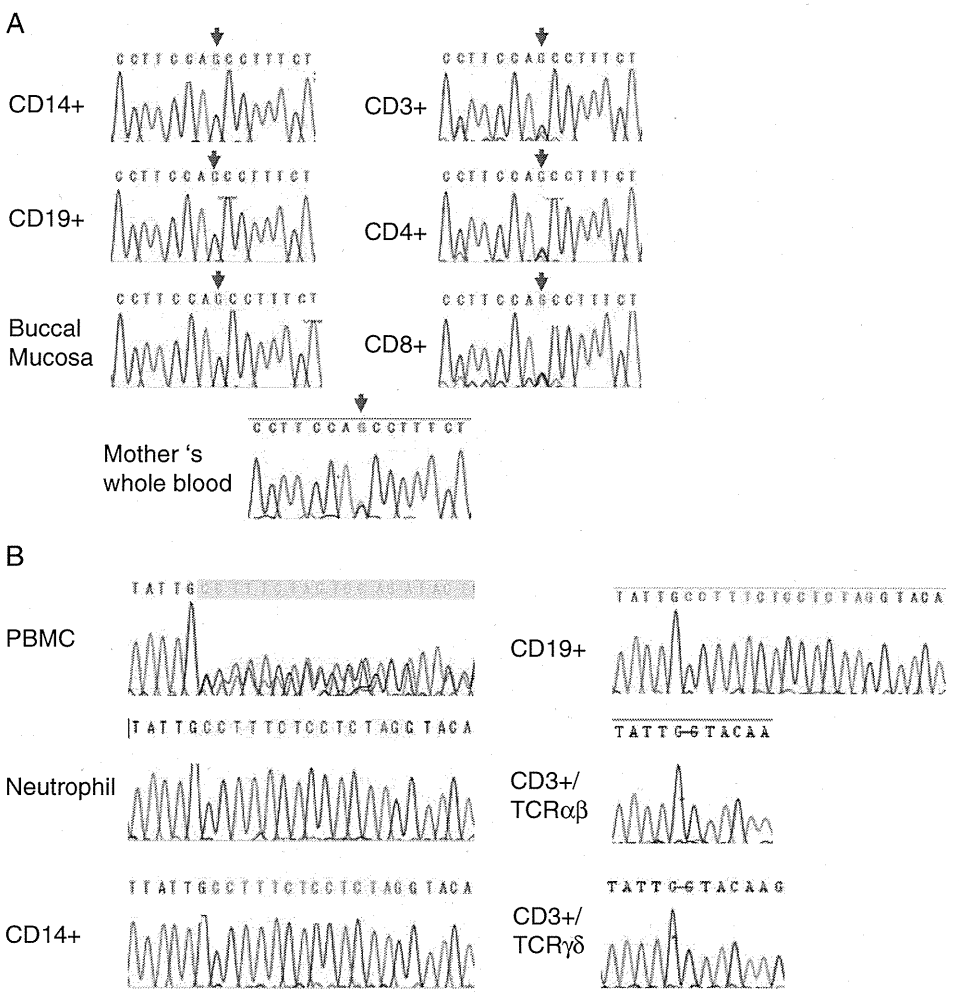

Fig. 2 Genetic analysis of various cell lineages at 9 years old. **a** Sequencing chromatograms of the patient's DNA from various immune cell lineages and buccal mucosa. *Red arrows* indicate the mutated base position c. 284–15. PBMCs from the patient's mother carried the same mutation. The patient's T cells show overlapping base changes at or around the mutated site. Data represent one of three independent experiments. **b** Sequencing chromatograms of the patient's cDNA from various cell types. Re characters indicate the inserted 14 bases spanning nucleotide −14 to nucleotide −1 of exon 3

subcloning and sequencing analysis of genomic DNA and cDNA was performed in various cell lineages. In B cells and monocytes, no reversion was detected and all of the cDNA clones had aberrant splicing (Table III). Analysis of TCRαβ+ cells revealed seven reversions, a true-back reversion, two fully compensating same-site reversions and four second-site reversions, all of which favored a functional reversion according to the splicing analysis software NNSPLICE0.9 [16] (Table IV). None of these base changes were detected in 200 clones from four healthy controls, indicating that the identified intron changes were unlikely to be due to PCR errors. The multiple reversions seen in this case differed from the single reversions seen in other reported cases of reversion mosaicism of SCID-X1 [2, 3]. One possible reason for this is that, compared with the previously reported exonic mutations, an intronic mutation is more likely to acquire additional reversions on top of a true-back mutation. Additionally, the nine-year lifespan of the patient may have provided increased opportunities for extra reversions to occur. TCRVβ V-to-DJ rearrangement is reported to be impaired in some SCID-X1 patients, suggesting that differentiation arrest occurs at the CD4 immature single positive (ISP) stage at which TCRVβ V-to-DJ recombination is completed in normal T cells [17]. Therefore, the

Table III Clonal analysis of *IL2RG* cDNA in various cell lineages

	CD3+	CD4+	CD8+	CD14+	CD19+
Wild-type cDNA	100 % (25/25)	100 % (31/31)	100 % (30/30)	0 % (0/45)	0 % (0/34)
Aberrant cDNA	0 % (0/25)	0 % (0/31)	0 % (0/30)	100 % (45/45)	100 % (34/34)

Data represent the percentages of wild-type or aberrant spliced cDNA subclones in each lineage. The ratio indicates the number of each clone as compared to the total number of clones analyzed, based on subcloning and sequencing analysis

Table IV Multiple additional mutations detected in subclones of the *IL2RG* gene

	Subclones	Mutations
Wild type	TT CCTCT T CCT T CCAACC	Wild type
Inherited mutation	TT CCTCT T CCT T CCAGCC	c.284-15A>G
No.1	TT CCTCT T CCT T CCATCC	c.284-15A>T
No.2	TT CCTCT T CCT T CCACCC	c.284-15A>C
No.3	TT CCTCT T CAT T CCAGCC	c.284-15A>G, c.284-21C>A
No.4	TT AGAGTGG CCTCT T CCT T CCAGCC	c.284-15A>G, c.284-29_284-28insAGAGTGG
No.5	TT CCTC CACCCGCCAAC	c.284-24_284-14del11ins CACCCGCCAA
No.6	TT CCTCT CAGCC	c.284-23_284-18delTCCTTC

reversions found in the patient's T cells must have occurred before or around the CD4ISP stage. Differences were observed in reversion genotypes between the TCRαβ+ cells and TCRγδ+ cells. TCRγδ+ cells had only one of the second-site reversions found in TCRαβ+ cells in addition to a true-back reversion (No.3 in Table V). The identification of fewer reversions in the patient's TCRγδ+ compared to TCRαβ+ cells may reflect the greater abundance of TCRαβ+ cells, increasing the likelihood of the stochastic occurrence of additional reversions [18]. Although no reversions in the patient's B cells or monocytes were observed, it is possible that the reversions occurred in the progenitor at the stage before commitment to T cells and may reflect the unique nature of T cell proliferation and expansion [19].

Reversion mosaicism has previously been reported in SCID-X1 patients with *IL2RG* mutations, but it was accompanied by reduced T cell number and low proliferative response to mitogens [2, 3]. The paradoxical nature of the patient's cellular immunity, a history of uneventful varicella infection, and the occurrence of widespread warts with very few naïve T cells prompted an evaluation of his T cell function. The TCR Vβ repertoire analysis of CD4+ and CD8+ T cells revealed comparable diversity to the normal controls (data not shown). CDR3 spectratyping analysis revealed the patient CD4+ T cells had as much variety as the normal controls, but his CD8+ T cells displayed restricted diversity (Fig. 3a). To evaluate the antigen-specific response of the patient's T cells, response to VZV was measured. The DTH reaction to subcutaneous VZV antigen and the IFN-γ production from VZV antigen-stimulated PBMCs measured by an ELISPOT assay were comparable to those of normal controls (Table I and Fig. 3b). These data suggested that the patient maintained normal cellular immune responses *in vivo* as well as having normal *in vitro* IFN-γ production ability against VZV antigen. Multiple reversions from intronic mutations could provide a sufficient number of normally functioning T cells and may improve the clinical phenotype compared to previously reported cases with single reversions. However, the number of TRECs in the patient's PBMCs (<10 copies/μg DNA) suggested a low level of recent thymic output, and the restricted diversity of TCRs observed in the patient's CD8+ cells might reflect the exhaustion of the T cell reservoir. To gain further insight, the long-term immunologic status of the patient was evaluated prospectively for 5 years. Absolute counts of CD4+ and CD8+ T cells as well as mitogen-induced T cell proliferation responses were measured every 2–5 months (Fig. 4). Unexpectedly, the number of both CD4+ and CD8+ T cells and mitogen-induced T cell proliferation responses were stable and the patient remained healthy over this period. In recent years, the effector memory subset of CD8+ T cells (CD8+/CD45RA+/CCR7-) has been taken as a marker of cell exhaustion or replicative senescence [20]. However, the majority of CD8+ T cells of the patient were memory CD8+ T cells (CD8+/CD45RA-/CCR7±) and the population of effector memory CD8+ T cells was very small (Table II). These data

Table V Clonal analysis of somatic mosaicism of the *IL2RG* gene in various cell lineages

	Wild type	Inherited mutation	No.1	No.2	No.3	No.4	No.5	No.6
TCRαβ+	21 % (7/33)	12 % (4/33)	9 % (3/33)	12 % (4/33)	21 % (7/33)	9 % (3/33)	6 % (2/33)	9 % (3/33)
TCRγδ+	2 % (1/42)	2 % (1/42)	0 % (0/42)	0 % (0/42)	95 % (40/42)	0 % (0/42)	0 % (0/42)	0 % (0/42)
CD3+	5 % (2/39)	5 % (2/39)	26 % (10/39)	13 % (5/39)	38 % (15/39)	3 % (1/39)	10 % (4/39)	0 % (0/39)
CD4+	32 % (25/79)	3 % (2/79)	13 % (10/79)	19 % (15/79)	16 % (13/79)	3 % (2/79)	3 % (2/79)	13 % (10/79)
CD8+	10 % (7/73)	4 % (3/73)	21 % (15/73)	19 % (14/73)	25 % (18/73)	8 % (6/73)	12 % (9/73)	1 % (1/73)
CD14+	0 % (0/33)	100 % (33/33)	0 % (0/33)	0 % (0/33)	0 % (0/33)	0 % (0/33)	0 % (0/33)	0 % (0/33)
CD19+	0 % (0/30)	100 % (30/30)	0 % (0/30)	0 % (0/30)	0 % (0/30)	0 % (0/30)	0 % (0/30)	0 % (0/30)

Data represent the percentages of each additional mutant subclone in each lineage. The ratio indicates the number of each mutant clone in various cell lineages as compared to the total number of clones analyzed, based on subcloning and sequencing analysis

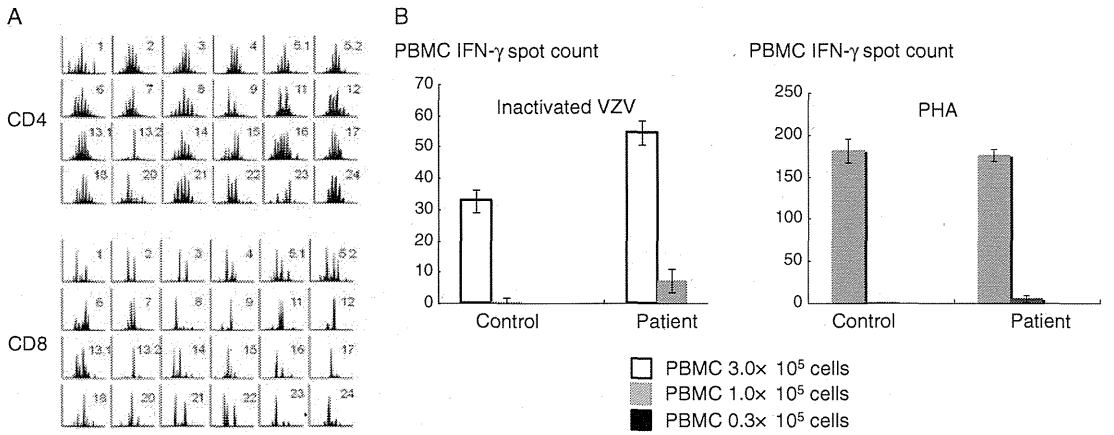

Fig. 3 Functional evaluation of T cells at 9 years old. **a** CDR3 spectratyping of the TCRVβ chain. Each TCRVβ fragment was amplified from cDNA with one of 24Vβ-specific primers (each Vβ chain is indicated). The size distribution of the PCR products was determined by an automated sequencer and GeneScan software. The CDR3 size distribution in CD4+ and CD8+ T cells from the patient is shown. **b** Elispot analysis of IFN-γ production as a measure of T cell function. (LEFT) Varicella-specific immune response to varicella zoster (VZV) antigen *in vitro*. Patient and control (from a healthy with a previous history of varicella infection) PBMCs ($0.3\text{-}3\times10^5$) were stimulated with inactivated VZV antigen or (RIGHT) PHA. Data are shown as mean ± SD

demonstrated that the patient maintained a certain level of T cell immunity for over a decade, despite the fact that the supply of fresh T cells from the thymus was limited and the patient suffered from generalized warts. Further follow up is required to determine if the patient can continue to maintain long-term T cell immunity.

In conclusion, this study indicates that it is critical to determine the NK cell number to avoid overlooking reversion mosaicism of SCID-X1. In addition, it has been shown that a number of *IL2RG* gene reversions can restore T cell functions and maintain T cell immunity against viral infection for at least 14 years.

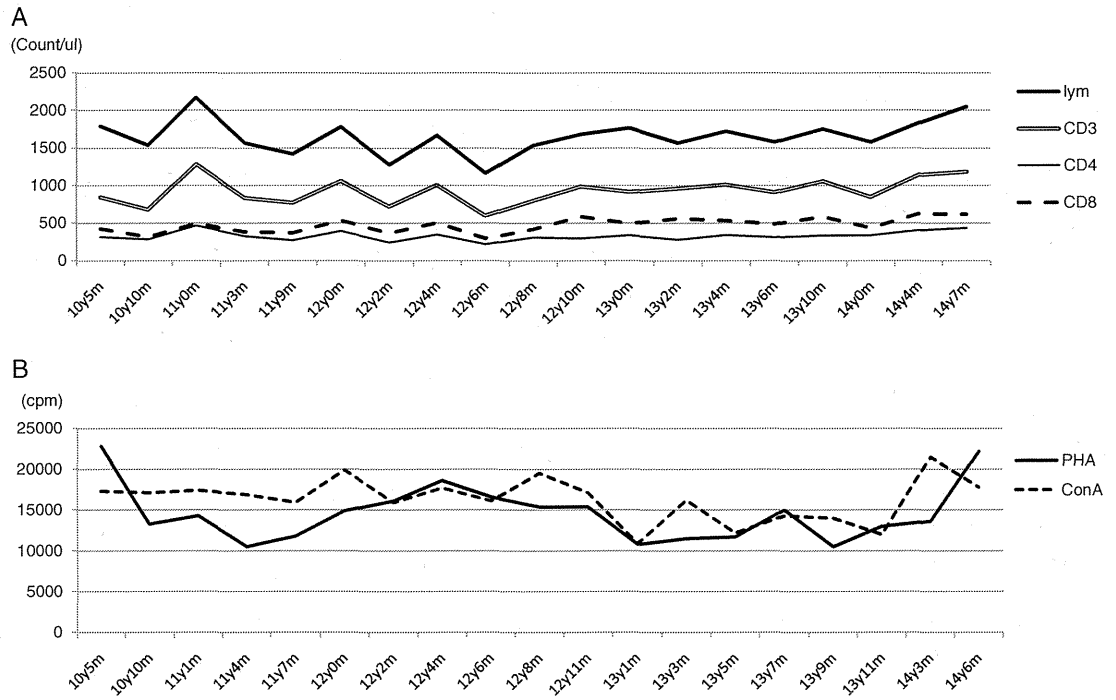

Fig. 4 Long term evaluation of T cell number and mitogen-induced proliferative response. **a** Absolute counts (per μl) of total lymphocytes (lym), CD3+ cells, CD4+ cells and CD8+ cells were measured for 4 years. **b** T cell proliferation in response to PHA (*solid line*) and Con A (*dotted line*). Healthy control values for PHA range from 20,500 to 56,800 cpm and for Con A from 20,500 to 65,700 cpm