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ORIGINAL ARTICLE

Fatal Enteroviral Encephalitis in a Patient with Common Variable Immunodeficiency Harbouring a Novel Mutation in *NFKB2*

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Abstract

Common variable immunodeficiency is the most prevalent of the primary immunodeficiency diseases, yet its pathogenesis is largely poorly understood. Of the cases that are monogenic, many arise due to pathogenic variants in *NFKB1* and *NFKB2*. Here, we report enteroviral encephalomyelitis as the cause of a fatal neurodegenerative condition in a patient with a novel heterozygous mutation in *NFKB2* (c.2543insG, p.P850Sfs36*) that disrupts non-canonical NF-κB signaling. Investigations of primary and secondary lymphoid tissue demonstrated a complete absence of B cells and germinal centers. Despite multiple negative viral PCR testing of cerebrospinal fluid during her disease progression, post-mortem analysis of cerebral tissue revealed a chronic lymphocytic meningoencephalitis, in the presence of Cocksackie A16 virus, as the cause of death. The clinical features, and progression of disease reported here, demonstrate divergent clinical and immunological phenotypes of individuals within a single family. This is the first reported case of fatal enteroviral encephalomyelitis in a patient with NF-κB2 deficiency and mandates a low threshold for early brain biopsy and the administration of increased immunoglobulin replacement in any patient with a defect in this pathway and deterioration of neurological status.

Keywords Immunodeficiency · CVID · NFKB2 · enterovirus · encephalitis · NF-kappaB2 deficiency

Introduction

Nuclear factor kappa B 2 (NF-κB2) is essential for the activation and differentiation of B cells, and development of secondary lymphoid organs [1]. Mutations affecting the C-terminus of the NF-κB2 protein result in common variable immunodeficiency (CVID) due to impaired activation of the alternative NF-κB pathway (CVID10, OMIM ID: 615577) [2–9]. The predominant feature reported in these patients with a deficiency of NF-κB2 function, is early-onset hypogammaglobulinemia. In the majority of cases, there is a reduction of isotype-switched memory B cells, and in a few, a complete absence of B cells. The function of other cells is also impaired; reduced $CD4^+$ T cell proliferation has been demonstrated in some [3, 4], NK-cell function has been shown to be defective [8], and reduction in the proportion of regulatory T cells and T follicular helper cells has been reported in several patients [4, 5]. In contrast, mutations leading to an apparent gain-of-function in NF-κB2 signaling, appear to cause a more severe immunodeficiency, characterized by susceptibility to Epstein-Barr Virus (EBV), and cytomegalovirus (CMV) infections [9].

Accompanying the abnormalities in immune cell types in patients reported with NF-κB2 deficiency is a susceptibility to endocrine disease, which is likely to be due to an autoimmune process in many cases, and alopecia areata and universalis.

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Defective central tolerance most likely plays a role in the pathogenesis of these autoimmune complications; reduction in expression of the autoimmune regulator (*AIRE*) has been observed in animal models of NF-κB2 deficiency [10, 11]. Furthermore, there is considerable overlap of clinical manifestations reported in individuals with NF-κB2 deficiency and those with autoimmune polyglandular syndrome-1 (APS-1 or APECED, OMIM ID: 240300) due to homozygous and compound heterozygous mutations of *AIRE* [12], including a recent report of anti-cytokine antibodies in a patient with NF-κB2 deficiency [13]. Loss of peripheral tolerance is also likely to be a significant contributing factor to autoimmune manifestations, with the observation of reduced numbers of circulating regulatory T cells in NF-κB2 deficient individuals [4].

Methods

Ethics and Informed Consent

Patients and their healthy relatives were recruited from the Department of Clinical Immunology and Allergy, Royal Melbourne Hospital, Victoria, Australia. Unrelated healthy donors were recruited from the Volunteer Blood Donor Registry, (VBDR). Informed consent was obtained from all individual participants included in the study for genomic analysis and functional immunologic studies prior to inclusion in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of Human Research Ethics Committees at Melbourne Health and The Walter and Eliza Hall Institute for Medical Research (Approved projects 2009.162, 10/02) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Genomic Analysis

Genomic DNA (gDNA) was extracted from whole blood from family members using QIAamp minikit (QIAGEN). Whole-exome sequencing was performed on gDNA from the proband and her sister (Illumina HiSeq 2000) at the Australian Genome Research Facility (AGRF, Melbourne). Analysis was performed as previously described [14]. Briefly, a filtering pipeline was applied to identify non-synonymous variants in protein-coding or splice-site regions with minor allele frequency of less than 1 % in publicly available databases (ESP6500; 1000 Genomes). Priority was given to frameshift and truncating variants. Additional in silico prediction tools were used for missense variants. Sanger sequencing was performed (AGRF) in mother and father to confirm familial segregation.

Cell Culture and Western Blotting

Prior to stimulation, peripheral blood mononuclear cells (PBMCs) were counted and seeded at 1×10^6 cells/mL, rested in RPMI 1640 supplemented with 1% FCS for up to 4 h at 37 °C, 5% CO_2 to synchronise cells into a resting state. Cells were then cultured at 37 °C, 5% CO_2 in tissue culture medium alone or stimulated by supplementing tissue culture medium with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (1 ng/mL), or CD40 ligand (100 ng/ml, MegaCD40L, Enzo Life Sciences), and cells were harvested at indicated times. Cell lysates, suspended in 6x Laemmli buffer, were loaded onto 4–12% BisTris polyacrylamide gels in MOPS buffer and transferred to a nitrocellulose membrane via the Bio-Rad Trans-Blot Turbo Transfer System, as previously described [14, 15]. Protein detection was performed using the following primary antibodies from Cell Signaling Technologies: phospho-p100 #4810; p100/p52, #4766; phospho-p105, #4806; p105/p50, #3035; phospho-p65; and p65, kit#9936, and detected with secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP, Santa Cruz Biotechnology). Images were obtained with the Bio-Rad ChemiDoc system and analysed using Image Lab software (Bio-Rad). Beta actin was used as a loading control.

Immunophenotyping

PBMCs were separated from whole blood and stained with the following antibodies according to standard protocols: BV650 anti-CD19 (BioLegend; clone HIB19); APC-H7 anti-CD27 (Miltenyi Biotec; MT271); BUV395 anti-CD3 (BD Pharmingen, SK7); PerCP Cy5.5 anti-CD4 (BD Pharmingen, RPAT4); PE anti-CD8 (BD Pharmingen, SK1); FITC anti-CXCR5 (BD Pharmingen, RF8B2); FITC anti-CD45RA (BD Pharmingen, HI100); PE anti-CD45RO (BD Pharmingen, UCHL1); PE-Cy7 anti-CD127(BD Pharmingen, AO19D5); BV421 anti-CD25 (BD Pharmingen, BC96); and BV711 anti-PD1 (BD Pharmingen, EH12.2H7). Samples were acquired on a five-laser BD Biosciences LSR Fortessa X-20 along with single-color compensation controls of antibody-conjugated beads (UltraComp eBeads, eBioscience), live/dead (FluoroGold) controls, and unstained controls.

Results

Clinical Presentation of Proband

The proband (II.1, Fig. 1a), the first-born child of non-consanguineous parents of northern European origin, was

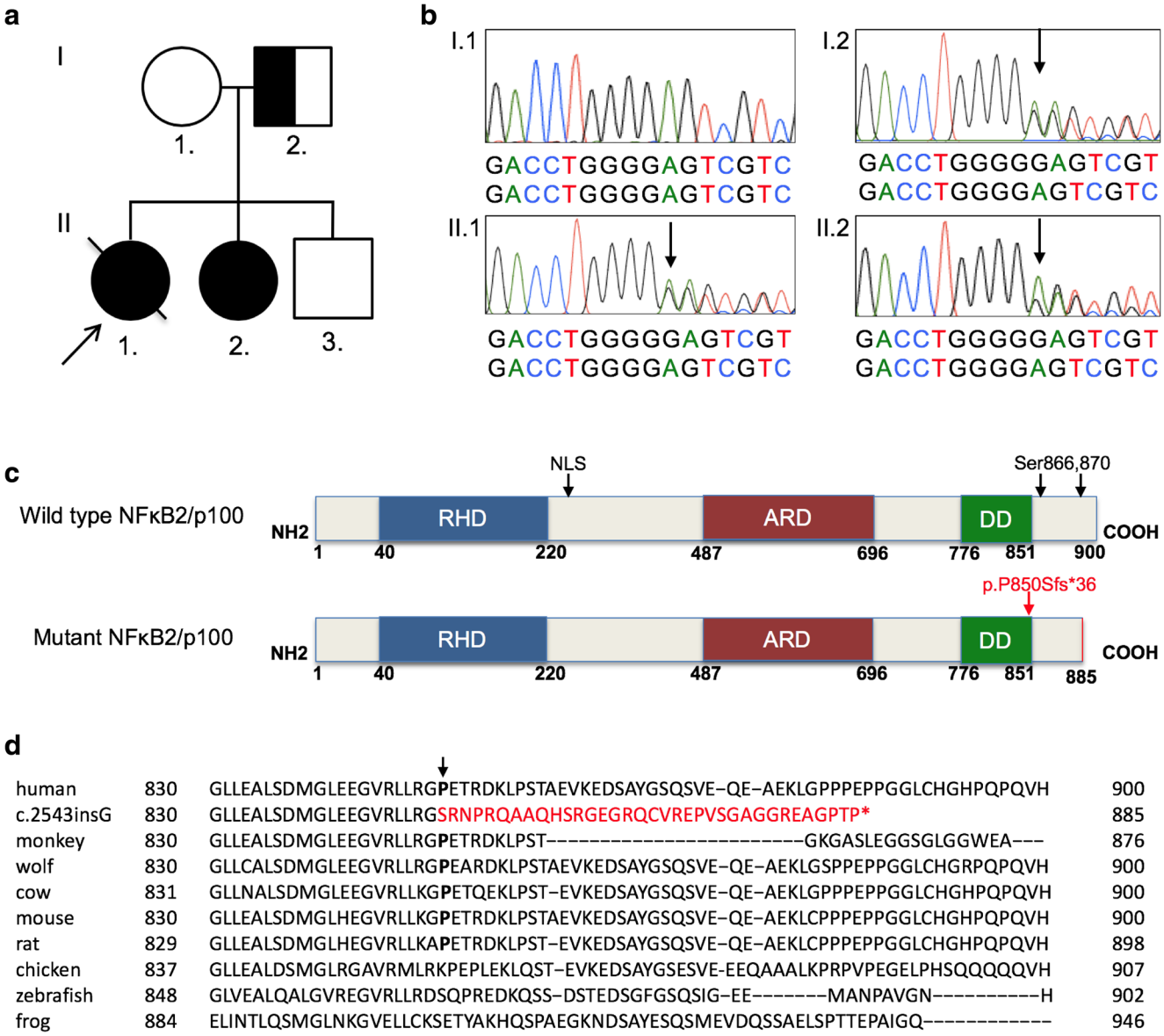


Fig. 1 Autosomal dominant inheritance of a novel *NFKB2* variant in a multiplex CVID family. **a** Pedigree of family with a heterozygous variant in *NFKB2* segregating with CVID in 2 affected sisters. The proband (II.2) is indicated by the arrow, and the line through shape indicates deceased. Shaded circles represent affected females, and unfilled circle represents unaffected female. Unfilled square represents an unaffected male, and the half-filled square represents unaffected male harbouring the *NFKB2* c.2543insG variant (I.2). **b** Sanger sequencing showing heterozygous insertion of G at position c.2543 (indicated by arrow) in exon 22 of *NFKB2* in the proband (II.1), her sister (II.2) and father (I.2), and wild type sequence in the mother (I.1). **c** Schematic illustration of NF-κB2 p100 wild type protein (top) and p.P850Sfs*36 mutant protein (bottom), depicting the following domains: NLS, nuclear localisation signal; RHD, rel homology domain; ARD, ankyrin repeat domain; DD, death domain. Ser866 and 870 indicate phosphorylation sites at serine residues, positions 866 and 870. The mutated residue, c.2543insG, p.P850Sfs*36, is indicated and is predicted to result in a truncated protein with loss of serine residues at positions 866 and 870. **d** Evolutionary conservation of NF-κB2, p100. The predicted protein sequence resulting from *NFKB2* c.2543insG (P850S) is depicted in red (mutalyzer). Arrow indicates the wild type P residue at position 850; conserved residues in bold

diagnosed with "possible CVID" in 1997, at the age of 9 years, after investigation for failure to thrive, recurrent lower respiratory tract infections and widespread molluscum contagiosum. Immunologic investigations at diagnosis revealed reduced IgG2 and IgA levels, reduced total B cell numbers, normal T and NK cell numbers, and normal T cell proliferative responses to PHA (phytohemagglutinin) (Table 1). Production of specific antibody in response to pneumococcal polysaccharide, conjugated tetanus and diphtheria vaccines, was absent. After an initial unsuccessful trial of continuous antibiotic therapy, intravenous immunoglobulin (IVIG) replacement therapy was introduced in 1998. She responded well to the treatment initially, with significantly fewer respiratory tract infections. Over time, there was also resolution of the molluscum contagiosum.

Table 1 Laboratory investigations of patients and family members

Family Member	II.1			II.2		I.1	I.2	II.3
Diagnosis/status	"Possible" CVID			CVID		Healthy	Healthy	Healthy
Date of test (month/year)	02/1997	10/2015		02/1997	10/2015	10/2015	10/2015	10/2015
IgG (5.5–16.3 g/L)	11.4	11*		*5.4*	10.7*	11.6	8.8	12.5
IgG2 (1.1–5.5 g/L)	*0.08*			*0.06*				
IgA (0.7–4.2 g/L)	*< 0.1*	*0.1*		*0.34*	*0.1*	1.9	2.5	1.8
IgM (0.3–2.9 g/L)	*0.27*	*0.1*		*0.3*	*0.1*	1.9	0.9	1.7
Lymphocyte count (1.2–4.0 × 10^9/L)	2.9	2.4		3.5	2.1	2.9	3.4	2.1
Total B cells, $CD19^+$ (% total lymphocytes, 7–23%)	*1%*	*absent*		7%	7.4%	15.5%	13.9%	–
Memory B cells, CD20+CD27+ (% total B cells, 16–55%)	–	*absent*		–	*absent*	27%	17.5%	–
Total T cells, $CD3^+$ (% total lymphocytes, 60–85%)	*57%*	63.4%		89%	82%	73.7%	68%	–
T helper cells, $CD3^+CD4^+$ (% total lymphocytes, 28–58%)	37%	39.3%		*64%*	*60.6%*	*59.3%*	50.9%	–
T regulatory cells, $CD3^+CD4^+$ $CD25^+$ $CD127^{lo}$ (%	–	–		–	*1.42%*	10.6%	7.22%	
TFH cells, $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ (%$CD4^+$ cells)	–	–		–	*0.17%*	4.02%	7.55%	–
Cytotoxic T cells, $CD3^+CD8^+$ (% total lymphocytes, 19–48%)	20%	24.9%		26%	21%	*12.3%*	19.6%	–
Natural Killer cells, $CD16^+CD56^+$ (% total lymphocytes, 6–29%)	*42%*	*34.3%*		*3%*	8%	7.5%	12.8%	–
Date of test (month/year)	2/2014	7/2014	3/2015	2000	2015	–	–	–
Cerebral MRI	NAD	Small foci of diffusion restriction	Diffuse cortical atrophy	NAD	NAD	–	–	–
CSF findings:				–	–	–	–	–
Lymphocytes	*24 × 10^6/mL*	*6 × 10^6/mL*	*4 × 10^6/mL*					
Polymorphonuclear cells	0 × 10^6/mL	*3 × 10^6/mL*	*9 × 10^6/mL*					
Erythrocytes	0 × 10^6/mL	*1 × 10^6/mL*	0 × 10^6/mL					
Protein (< 0.45 g/L)	*0.48 g/L*	*0.54 g/L*	*0.58 g/L*					
Glucose (2.2–3.9 mmol/L)	3.5 mmol/L	2.7 mmol/L	3.0 mmol/L					
Gram stain, ZN stain, cryptococcal antigen, and PCR[¶]	All negative	All negative	All negative					
Autoantibodies: anti-GAD antibodies (<5U/ml)	*(242 U/mL)*	–	*177 U/mL*	–	*60 U/mL*	–	–	–

Units of measurement and reference ranges in parentheses; CSF, cerebrospinal fluid; *MRI*, magnetic resonance imaging; *CVID*, common variable immunodeficiency; *NAD*, nothing abnormal detected; *PCR*, polymerase chain reaction; *GAD*, glutamic acid decarboxylase; *lymph*, lymphocytes; *PMNs*, polymorphonuclear cells; *ZN*, Ziehl-Nielsen; *TFH*, T follicular helper [¶] PCR was performed for herpes simplex viruses-1 and -2, varicella zoster virus, herpes zoster virus, enterovirus, parechovirus ,and polyoma (JC and BK) viruses; italic indicates abnormal values; *on intravenous immunoglobulin replacement; −, not performed

Clinical Features of Other Family Members

The proband's younger sister (II.2, Fig. 1a), aged 7, was also diagnosed with CVID after a similar history of respiratory tract infections and reduced total IgG, IgG2, and IgA levels (Table 1). At the age of 11, she was diagnosed with hypothyroidism and central adrenal insufficiency. At 13, she developed autoimmune thrombocytopenia, which was treated successfully with rituximab after an initial lack of response to corticosteroids and IVIG.

At diagnosis, and at long-term follow-up after treatment with rituximab, the younger sister had normal total B cell numbers, although there was an absence of memory B cells (Table 1). Apart from a reduced proportion of NK cells at diagnosis, all other lymphocyte populations tested were normal in number (Table 1). However, more extensive immunophenotyping revealed a reduction in the proportions of T follicular helper cells and T regulatory cells (Table 1).

The parents and younger brother were healthy. In particular, their immunoglobulin levels and proportions of B and T lymphocytes were normal, whereas the proband developed a complete absence of peripheral B cells (Table 1).

Clinical Progression of Proband

In February 2014, at the age of 27 years, the proband presented with a 2-month history of weight loss, anorexia, headache, and visual disturbance. She had been receiving 0.5 mg/kg monthly Intragam P™ (Commonwealth Serum Laboratories, CSL Behring, Australia) with adequate trough levels between 8 and 9 g/L. Neurologic examination revealed no focal deficits; however, a vitreal hemorrhage was observed by fundoscopy. Cerebrospinal fluid (CSF) was notable for the presence of lymphocytes (24×10^6/mL) in the absence of erythrocytes and polymorphonuclear cells (Table 1). CSF bacterial culture and PCR for herpes simplex viruses (HSV-1, HSV-2), herpes zoster virus (HZV), varicella zoster virus (VZV), polyoma (JC and BK) virus, and entero- and parechoviruses were negative (Table 1). Cryptococcal antigen was not detected, and there was no evidence of mycobacteria by Ziehl-Nielsen staining and PCR. Cerebral imaging by CT and MRI was normal (Fig. 2a). She was treated with IV benzylpenicillin for 72 h, given the possibility of bacterial meningitis; however, after review of the results of CSF analysis, a presumptive diagnosis of aseptic meningitis was made. Despite an initial resolution of the symptoms, 6 months later, the patient represented with a recurrence of headaches. No additional new neurologic features were present. Further imaging and repeat analyses of CSF revealed no significant abnormalities (Table 1).

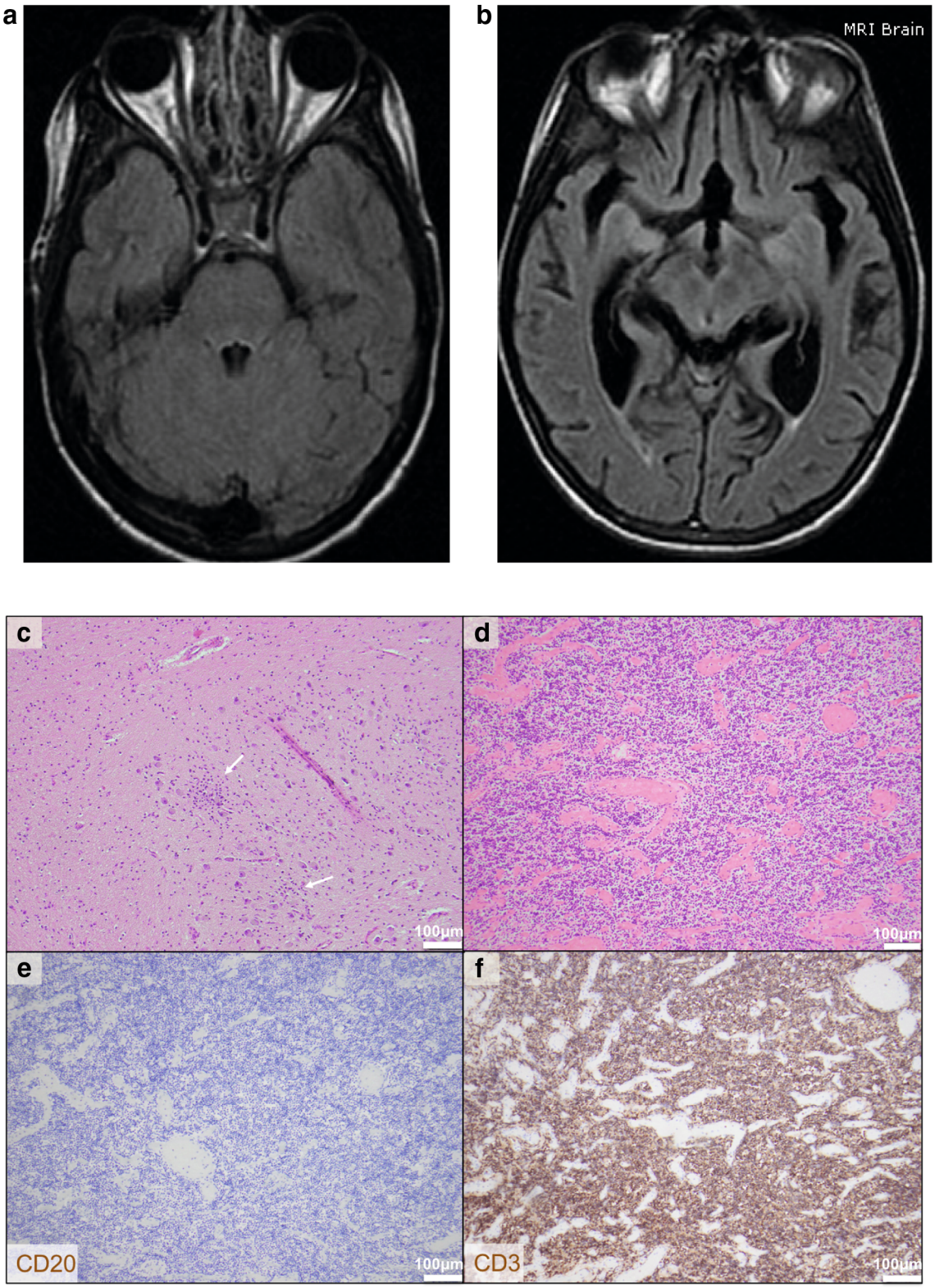
Twelve months after the initial presentation, the proband returned to the clinic with significant neurologic deterioration. Her family reported personality changes characterized by social withdrawal, and impaired memory, which had developed over a 1-month period. Neurological examination revealed an unsteady broad-based shuffling gait, dysmetria, and dysarthria. Tone and reflexes were generally increased. MRI revealed inflammation of bilateral mesial temporal lobes and cerebellum (Fig. 2a), likely accounting for the personality and gait changes respectively. Serum and CSF testing for antineuronal antibodies was negative, although anti-GAD antibodies were detected in serum (Table 1). Brain biopsy was recommended, but not performed due to the patient and family's wishes.

On the basis of these clinical features, the abnormalities on cerebral imaging, and lack of evidence of an infectious cause, a preliminary diagnosis of autoimmune encephalitis was made. High-dose IVIG (2 g/kg) and methylprednisolone 2 mg/kg was administered; however, the patient's condition continued to worsen. She was unable to ambulate, had significantly impaired vocalization and swallowing had become uncoordinated. A single dose of cyclophosphamide (500 mg) was administered, but the patient's condition deteriorated further over the following 2–3 weeks, and she died 4 weeks after withdrawal of active treatment.

At post-mortem, a chronic lymphocytic meningoencephalitis was diagnosed, with a T lymphocyte-rich, perivascular and parenchymal lymphocytic infiltration, neuronophagia and microglial upregulation, most prominent in the brainstem and cerebellum (Fig. 2c). No specific viral inclusions or viral immunoreactivity was found for HSV, HZV, CMV, measles virus (MeV), or polyoma virus. Real-time PCR detected enteroviral RNA from cerebral tissue, which was subsequently identified by Sanger sequencing as Coxsackie A virus, subtype A16. Reticulo-endothelial system changes included an absence of $CD20^+$ B lymphocytes in the splenic lymphoid tissue and an absence of germinal centers and $CD20^+$ B lymphocytes in sampled lymph nodes, as detected by histological analyses (Fig. 2d–f). The bone marrow was normocellular, the thymus normal for age, and the pituitary was normal. In addition to the encephalitis, bacterial bronchopneumonia, pulmonary edema, multiple renal cortical adenomata, and multiple plantar verrucae were also noted.

Identification of Novel *NFKB2* Mutation by Whole-Exome Sequencing

In order to identify the genetic etiology of the clinical phenotypes in this family, we performed whole-exome sequencing of gDNA from the proband and her younger sister, as described above. We identified a heterozygous insertion of a guanine at c.2543 in exon 22 of *NFKB2*. Sanger sequencing confirmed that the variant segregated with disease in both sisters, and was also identified in the



healthy father (Fig. 1b). The variant is predicted to lead to disruption of the phosphorylation sites required for initiating degradation of p100 to its active subunit, p52 (Fig. 1c). Proline at amino acid position 850 is highly conserved in mammals and insertion of G at c.2543 and results in proline to serine substitution and the creation of a premature stop codon, 36 residues along the resulting protein (Fig. 1d).

◀ **Fig. 2** NF-κB2 deficiency is associated with reduced B cell numbers, absence of germinal centers, and the development of enteroviral encephalitis in the proband. Cerebral MRI of proband from (**a**) February 2014 is normal in appearance, and, in (**b**) March 2015 shows marked cerebral atrophy. **c** Histology of cerebellar dentate nucleus shows features of encephalitis including perivascular and parenchymal lymphocytic infiltration (arrows) with neuronophagia and upregulated microglia (Hematoxylin and Eosin, H&E, × 100 actual magnification). (**d–f**) Post-mortem of carinal lymph node reveals (**d**) small lymphoid cells (H&E × 100 actual magnification), demonstrating complete absence of lymphoid follicles and germinal center architecture, (**e**) CD20 immunoperoxidase staining (positive staining, brown; negative, blue) depicting a complete absence of B lymphocytes (× 100 actual magnification), and (**f**) a dominant population of small T lymphocytes (CD3 immunoperoxidase, positive staining, brown; × 100 actual magnification)

Immunophenotyping Identifies a Variable B Cell Defect Associated with NF-κB2 Deficiency, with a Reduction in T Follicular Helper Cells and Regulatory T Cells in the Surviving Sister

Given the divergent clinical history of these patients, we sought to compare the proportions of B and T cell subsets in this kindred to that of a cohort of CVID patients ($n = 16$) and unrelated healthy donors ($n = 35$). Consistent with clinical phenotyping previously performed, the deceased sister had a complete absence of peripheral B cells (0.11% of total lymphocytes), whereas her sister had a reduced proportion of B cells (3.83%; Fig. 3a). Although no significant differences were observed in the proportion of B cells between

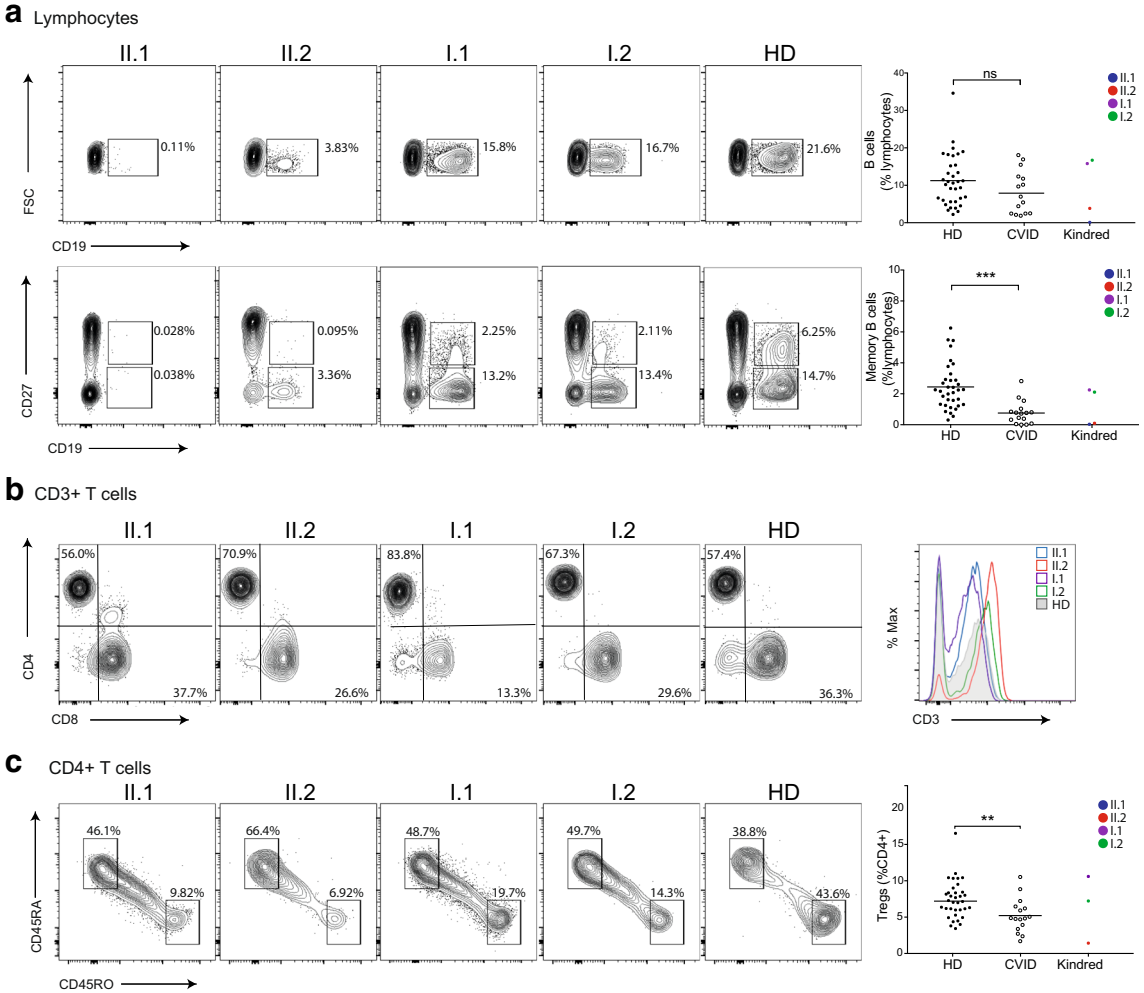


Fig. 3 Immunophenotyping reveals a variable B cell defect and reduced T follicular helper cells and regulatory T cells in NF-κB2 deficiency. Immunophenotyping of PBMCs depicting the proportion of (**a**) total B cells ($CD19^+$), naive B cells ($CD19^+CD27^-$) and memory B cells ($CD19^+CD27^+$). **b** Helper ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) and (**c**) naïve ($CD4^+CD45RA^+$) and memory helper T cells ($CD4^+CD45RO^+$). Family members are labeled according to pedigree in Fig. 1. HD indicates healthy donor. Representative flow plots are shown for each family member tested, and an unrelated healthy donor (HD) and summary graphs are shown for HD ($n = 35$), unrelated CVID patients ($n = 16$) and family members from kindred as indicated. (ns, non-significant; *, p<0.05; **, p<0.01; ***, p<0.005)

healthy donors and a cohort of CVID patients, the affected sister and their parents were within the lower and upper limits of the group, respectively. Not surprisingly, a significant reduction in the percentage of memory B cells was observed in the CVID group, compared to healthy donors, again with the affected individuals showing an almost complete absence of memory B cells and normal proportions of memory B cells observed in their unaffected parents. While total helper ($CD3^+CD4^+$) and cytotoxic ($CD3^+CD8^+$) T cells were normal in all family members (Fig. 3b), a reduction in memory T helper cells and regulatory T cells was observed in affected sisters, but not in their parents, or unrelated healthy donor (Fig. 3c).

Functional Validation of the *NFKB2* Variant Demonstrates Impaired Phosphorylation of p100 after Short-Term Stimulation

To determine the functional effect of the *NFKB2* variant on NF-κB signaling, we next performed western blot analysis of lysates from PMA/ionomycin-stimulated PBMCs isolated from II.2 and an unrelated healthy donor at both early (10–20 min), and later (72 h) timepoints, given the biphasic expression of phospohorylated p100 following stimulation (Fig. 4a). Phosphorylation of p100 was severely reduced in stimulated PBMCs from II.2, compared to an unrelated healthy donor, with almost no protein detected after 20 min of stimulation (Fig. 4b). After 3 days, however, the amount of

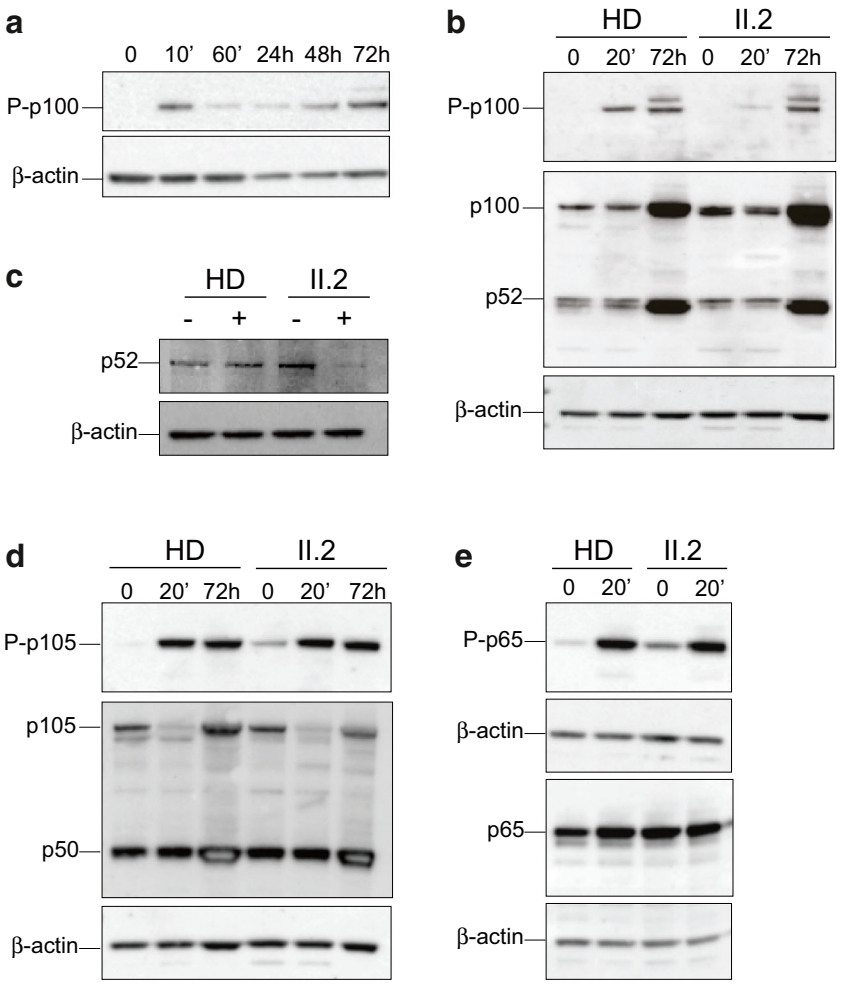


Fig. 4 A defect in early activation of non-canonical NF-κB signaling is observed in the presence of *NFKB2* c.2543insG. **a** Kinetics of phosphorylated p100 (P-p100) expression following stimulation with PMA/ionomycin demonstrates biphasic expression both at early and later timepoints. **(b)** Western blot of PBMCs from the patient, II.2, and a healthy donor, either unstimulated, or stimulated with PMA/ionomycin, for indicated times to measure early and late activation of NF-κB signaling. Proteins detected to measure non-canonical signaling are phosphorylated full-length p100 (P-p100) and expression of full-length p100 and p52 subunit. **c** Expression of p52 active subunit was evaluated in PBMCs from II.2 and another unrelated healthy donor either unstimulated or following incubation with CD40L for 48 h as indicated (**d**, **e**). To determine the effect on canonical signaling, phosphorylation of p105 (P-p105) and p65 (P-p65) as well as expression of p105, p50, and p65 was measured as indicated. Beta-actin was used as a loading control. Western blot analysis was performed in four independent experiments

phosphorylated p100 was comparable to the healthy donor and only a marginal reduction in processed p52 was observed. Both the full-length p100 and p52 subunit were detected in the affected individual and healthy donor. However, in II.2, expression of p100 was uniformly higher than in healthy donor individuals, suggesting an accumulation of non-processed p100 protein over time. A defect in p52 processing was also observed following CD40L activation (Fig. 4c), further supporting a functional defect in NF-κB2 signaling and functional p52 deficiency. The canonical pathway appeared unaffected, as no differences were observed for p105/p50 expression and signaling via the NF-κB1 pathway was comparable to healthy donor (Fig. 4d, e). Together, these results suggest that *NFKB2*^{P850Sfs36*} impairs NF-κB2 signaling at early timepoints, but not under prolonged stimulation conditions, and this may result in a reciprocal accumulation of p100, but does not affect canonical signaling.

Discussion

CVID is a disease of complex pathogenesis, which in the majority of cases is poorly understood. Monogenic causes account for a minority; however, the implementation of whole-exome and whole-genome sequencing continues to expand our understanding of the mechanisms by which this condition may develop [16]. Fourteen monogenic causes have been identified according to OMIM, of which NF-κB2 deficiency is one. We have previously discovered mutations in *NFKB1,* which cause CVID due to haploinsufficiency of the active subunit of the canonical NF-κB1 signalling pathway, p50 [15]. Together, *NFKB1* and *NFKB2* mutations are likely to be the predominant monogenic causes of CVID, particularly as they lead to autosomal dominant inheritance of the condition [17]. One of the features of dominantly inherited conditions is a variable penetrance of clinical features, as is reflected in this family. While certain features of monogenic CVID are common to all forms of the disease, polygenic and monogenic alike, some complications are restricted to single genes or pathways, such as the adrenocorticotropic hormone (ACTH) deficiency and alopecia seen in patients with *NFKB2* mutations affecting the C-terminal component of the protein, leading to disease by a dominant negative mechanism. In fact, more than half of the patients so far reported with NF-κB2 deficiency due to dominant negative mutations were reported to have ACTH deficiency and several have alopecia areata or totalis (Table 2) [2–9, 13]. Interestingly, alopecia is also a feature of NF-κB1 deficiency, but ACTH deficiency has not been reported in these individuals [15, 17, 18]. Here, we demonstrate the normal histologic appearance of the pituitary and thymus in a patient with NF-κB2 deficiency, in addition to complete absence of B cells and germinal centers in secondary lymphoid tissue (Fig. 2d–f). We have also demonstrated an impairment of activation of non-canonical NF-κB signaling after both PMA/ionomycin and CD40L stimulation, as evidenced by reduced phosphorylation of p100 early after activation, with a corresponding accumulation of total p100 and reduced active p52 (Fig. 4b,c) with canonical signalling largely intact (Fig. 4d,e). Together, these findings suggest a functional insufficiency of p52. The frameshift mutation identified in this family, while novel, is only 3 amino acids upstream from the most commonly reported *NFKB2* mutation, namely the nonsense mutant, p.Arg853*, and 5 amino acids from the first frameshift mutation reported, p.Lys855Serfs7* [2, 6, 8, 13, 19]. Furthermore, reported pathogenic *NFKB2* mutations in the NIK domain, downstream of the P850Sfs*36 mutation described here, are restricted to damaging only a single phosphorylation site (p.A867Cfs19*) [3], or located immediately adjacent to a phosphorylation site (D865G) [4, 6], and thus may be predicted to have a less pronounced effect on p100 phosphorylation than the mutation in the current report, which disrupts both Ser866 and Ser879 sites. Our observation of accumulated p100 following long-term stimulation in patient cells also supports disruption of impaired p100 processing to p52 in these individuals. Thus, the findings reported here are likely to be broadly applicable to patients with other dominant negative *NFKB2* mutations.

Autoimmune complications affect at least 25% of individuals with CVID [20] and pose therapeutic challenges due to the risk of immunosuppression in individuals with pre-existing immunologic incompetence. This family, with three phenotypically diverse individuals with the same *NFKB2* mutation, demonstrates the complexity of CVID, even when a monogenic etiology is found. The more widespread application of genomic technologies for the diagnosis of CVID, and other PIDs, will continue to reveal the spectrum of phenotypes associated with heterozygous disease-causing variants. The term monogenic is more appropriate here than Mendelian, as penetrance and expressivity are clearly variable. A pertinent example is CTLA4 haploinsufficiency, with a recent large cohort study revealing a clinical penetrance of 67% [21]. This is intriguing and raises many questions for future studies regarding the impact of genetic modifiers, in particular with respect to the contribution of environmental exposures and the potential for epigenetic effects.

Cocksackie A16 virus is a ubiquitous enterovirus, typically causing self-limited hand, foot, and mouth disease in children, although in rare cases it may lead to neurologic disease even in ostensibly immunocompetent individuals [22]. It is likely that most individuals are exposed to the virus and recover without serious adverse outcomes. The absence of B cells and immunoglobulin leads to an increased susceptibility to infection; enteroviral encephalitis is recognized as a potential complication of X-linked agammaglobulinemia (XLA); however, most reported cases have occurred in the setting of limited or no immunoglobulin replacement [23–25]. Adequate

Table 2 Clinical features of patients with dominant negative *NFKB2* mutations

NFKB2 variant protein change [Ref]	c.2564delA K855Sfs* [2, 7]	c.2557C > T R853* [2, 8, 13]	c.2593_2600del, D865Vfs*17 [5]	c.2598_2599insT, pA867Cfs*19 [3]	c.2594A > G, p.D865G [4, 6]	c.2600C > T p.A867V [6]	c2556_2563 p.R853Afs*29 [6]	c.2563A > T, p.R853* [6]	c.2596A > C p.S866R [9]	c.2543insG, p.P850Sfs*36 (current report)
Onset (years)	< 10, 1.5, 2	< 2, 2, 2, 23	1, 0.5	1	11, 20, 2, ?	8, 7	4, ?	2	8	2, 3
Diagnosis (years)	30, 6, 3	10, 4, 2, 23	9, 7	1.5	40, 20, 5, ?	9, 7	4, ?	18	10	7, 9
ACTH deficiency	3/3	3/4	1/2	0/1	1/4	1/2	1/2	1/1	1/1	1/2
Alopecia	1/3	4/4	0/2	1/1	3/4	0/2	0/2	1/1	0/1	0/2
Trachyonychia	1/3	4/4	0/2	1/1	0/4	0/2	0/2	1/1	0/1	2/2
Autoantibodies	2/3	4/4	0/2	0/1	0/4	0/2	0/2	1/1 (anti-IA2)	0/1	2/2 (anti-GAD)
Neurologic disease	Bell's palsy 1/3	Aseptic meningitis 1/4	CNS inflammation[¶] 1/2	0/1	Aseptic meningitis 1/4	0/2	0/2	0/1	0/1	Enteroviral encephalitis 1/2
Skin disorder	Vitiligo 1/3	MC 1/4 ED 3/4 psoriatic rash 1/4	0/2	0/1	Aquagenic urticaria 1/4	0/2	0/2	ED 1/1	MC 1/1	MC 1/2 psoriatic rash 1/2 BCC 1/2 plantar warts1/2

(ACTH, adrenocorticotropic hormone; *CNS*, central nervous system; *GAD*, glutamic acid decarboxylase; *IA2*, islet antigen 2; [¶] unspecified cause; *ED*, ectodermal dysplasia; *MC*, molluscum contagiosum; *BCC*, basal cell carcinoma;*?*, uncertain)

immunoglobulin replacement is usually sufficient to prevent the development of chronic infection. In this reported case, trough immunoglobulin levels were maintained between 8 and 9 g/L throughout the initial course of disease, and were above 10 g/L in the last 3 months of illness (Table 1). Other reports of disseminated enteroviral infections related to B cell depletion for the treatment of hematological malignancies and autoimmune disease provide further evidence that B cells provide a role other than antibody secretion in their defence against this infection [26, 27]. A single case of chronic enteroviral infection has been reported in a patient with BLNK deficiency, an extremely rare cause of autosomal recessive agammaglobulinemia (OMIM ID: 613502), and was associated with arthritis and dermatitis, in the absence of neurologic involvement [28]. Another report of a young female patient, who only months after receiving a diagnosis of CVID succumbed to enteroviral meningoencephalitis (Cocksackie A16 virus), highlights the necessity to be vigilant for this potential diagnosis [29]. It is unclear whether any genetic investigations were performed in this case, and B cell numbers and immunoglobulin levels were not reported.

Due to the ubiquitous nature of enteroviruses, immunoglobulin derived from multiple healthy donors should provide protection against most enteroviral subtypes. The use of purified, specific immunoglobulin has been implemented in other chronic viral infectious disease, such as cytomegalovirus [30], although no enteroviral specific product is currently available. An inactivated EV71 vaccine has been demonstrated to be effective in protecting against hand, foot, and mouth disease in a large cohort of infants and children in China, but was not effective in reducing severe cases of disease, and offered no protection against Cocksackie A16 infection [31]. Other therapeutic measures have been attempted in isolated cases of enteroviral encephalitis, including high-dose IVIG and intraventricular immunoglobulin, with variable, but predominantly poor outcomes [23, 32]. The use of ribavirin and pleconaril, which have in vitro activity against enterovirus [33], has not been well studied in human enteroviral infections. Other agents used as adjunctive therapy to IVIG include fluoxetine, which has been successfully used in a single case of an XLA patient with enteroviral encephalitis [34], and itraconazole, which has also been demonstrated to have in vitro anti-enteroviral activity [35].

Differentiating autoimmune disease from infections in immunodeficiency is crucial to avoid outcomes such as that reported here. A recently reported case of a CVID patient, who was also being treated with mycophenolic acid for lymphocytic interstitial pneumonitis and adalimumab for colitis, is suggestive of autoimmune demyelination with no evidence of infection on brain biopsy or deep metagenomic sequencing of CSF for potential pathogens [36]. From the current report, it is clear that the sensitivity of PCR from CSF samples may be insufficient for detection of infectious etiologies of encephalitis, including enterovirus. In our patient, viral PCR was performed on CSF samples on three separate occasions (February 2014, July 2014, March 2015; Table 1), and enterovirus was only amplified from a direct sample of brain tissue at autopsy, likely due to the greater sensitivity of testing a tissue sample. Unfortunately, a brain biopsy was declined during the course of the patient's illness. Several other cases of presumed autoimmune encephalitis in CVID patients are reported, including anti-GAD antibody associated limbic encephalitis developing as the presenting feature of CVID [37]. Detection of low-titer anti-GAD antibodies in the case of our patient (Table 1), when interpreted with the cerebral MRI (Fig. 2b), was an additional factor in the diagnostic challenge which arose, but the clinical significance of these autoantibodies, if any, remains unclear.

Conclusions

The challenge of differentiating autoimmune disease from infection is not new to physicians caring for patients with CVID, nor other immunosuppressed individuals with pre-existing autoimmune disease. Awareness of the particular risk of enteroviral infection, a low threshold for early brain biopsy, and administration of therapy with high-dose IVIG and antiviral therapy should be strongly considered in any patient with CVID and deterioration of neurological status.

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Compliance with Ethical Standards

Patients and their healthy relatives were recruited from the Department of Clinical Immunology and Allergy, Royal Melbourne Hospital, Victoria, Australia. Informed consent was obtained from all individual participants included in the study for genomic analysis and functional immunologic studies prior to inclusion in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of Human Research Ethics Committees at Melbourne Health and The Walter and Eliza Hall Institute for Medical Research (Approved projects 2009.162, 10/02) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest The authors declare that they have no conflict of interest.

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