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The importance of functional validation after next-generation sequencing: evaluation of a novel *CARD11* variant

To the Editor:

The advent of next-generation sequencing (NGS) techniques such as whole exome sequencing (WES) has greatly accelerated the identification and treatment of rare Mendelian diseases. In particular, these approaches have demonstrated immense utility for identifying inborn errors of immunity (primary immunodeficiencies [PIDs]). This impact is emphasized by the fact that of the estimated 350 gene defects linked to inborn errors of immunity, ~100 have been described since the implementation of NGS techniques in 2010.¹

Within the broad spectrum of inborn errors of immunity, the combined immunodeficiency disorders (CIDs) are caused by a group of gene defects that result in dysfunctional lymphocyte activation. Nuclear factor- κ B (NF- κ B) is a central regulator of lymphocyte activation, proliferation, and differentiation. Its activation is facilitated by the assembly of the caspase recruitment domain family member 11 (CARD11)—B-cell CLL/lymphoma 10 (BCL10)—MALT1 paracaspase (MALT1) (CBM) signalosome.² Confirming the critical role of the CBM complex in immunity, patients have now been identified with mutations in each of the three CBM components, resulting in a unifying

phenotype of aberrant NF- κ B activation, abnormal T- and B-cell development and function, and recurrent infections.² CARD11 is unique in the range of “CARD11-opathies” that are caused by the three categories of germline mutations identified in this gene: (i) homozygous loss-of-function mutations causing combined immunodeficiency³; (ii) heterozygous gain-of-function mutations leading to “B cell expansion with NF- κ B and T cell anergy” (BENTA) disease⁴; and (iii) heterozygous CARD11 mutations with dominant-interfering activity resulting in severe atopic disease.⁵ Here, we report the functional impact of a homozygous CARD11 variant (c.1798G>T, p.D600Y).

The index patient is an 11-year-old Canadian First Nations boy born to unrelated parents. Immediate family history was unremarkable, with the exception of his mother, who was diagnosed with rheumatoid arthritis at age 21. His two younger sisters and one younger brother are all reported to be healthy, while an older brother died from an accidental injury (Figure 1A). Infectious history was notable for several episodes of oral candidiasis until age 2 years, an episode of pneumonia requiring hospitalization, and a prolonged episode of diarrhea positive for *Cryptosporidium* spp. He suffered

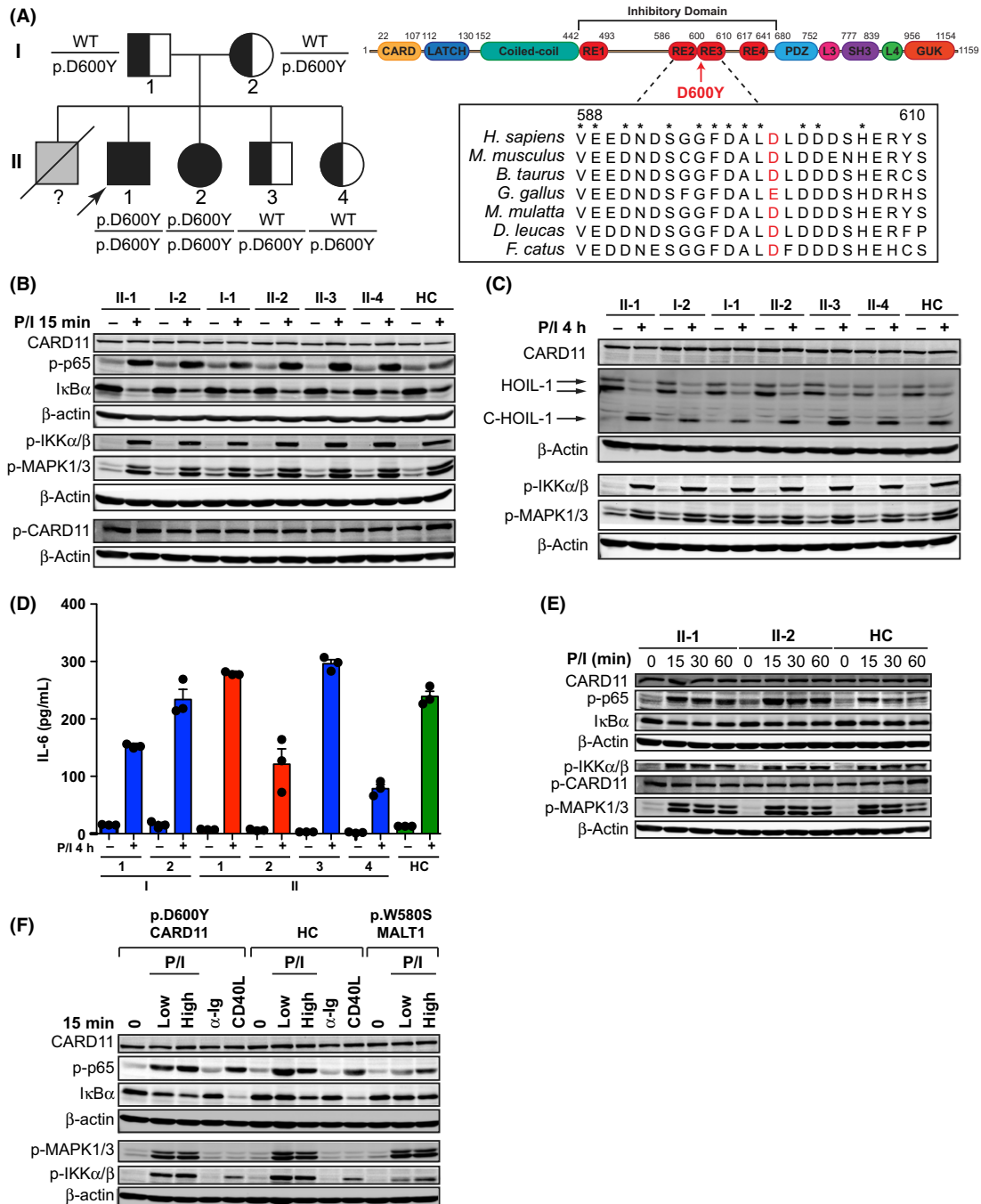


FIGURE 1 Functional characterization of the D600Y CARD11 variant in patient and family B cells. **A, Left panel:** Family pedigree constructed from sequencing whole blood from the patient and each family member. The gray square with a line struck through indicates a deceased older brother with no available samples to test. Arrow refers to index patient. Genotypes are indicated beside/below each individual. **Right panel:** Schematic representation of the CARD11 protein domains. Red arrow indicates p.D600Y substitution site. Inset shows enlargement of substitution region and site (shown in red) aligned to corresponding regions in other species. CARD, caspase-associated recruitment domain; RE, repressive element; PDZ, postsynaptic density protein (PSD95), *Drosophila* disk large tumor suppressor (Dlg1), zonula occludens-1 protein (zo-1) domain; L3, linker 3; L4, linker 4; GUK, guanylate kinase domain. **B-C,** Immunoblotting of LCLs stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ M/L ionomycin (P/I) for 15 min (B) or 4 h (C). **D,** IL-6 quantification by ELISA for LCLs stimulated for 4 h with P/I. Blue bars indicate heterozygous individuals, red bars indicate homozygous individuals, and green bars indicate an individual with wild-type CARD11 genotype. **E,** LCLs from two patients homozygous for the D600Y variant (the index patient and his sister) were stimulated with P/I in a time course ranging from 0 to 60 min and compared to a healthy control. **F,** Immunoblot of LCLs stimulated with a low dose of P/I (25 ng/mL PMA + 0.5 μ M/L ionomycin), a high dose of P/I (50 ng/mL PMA + 1 μ M/L ionomycin), α -Ig (10 μ g/mL anti-IgM+IgG), or CD40L (10 μ g/mL)

from recurrent otitis media and has had chronic otorrhea intermittently positive for *Pseudomonas* spp. or *Achromobacter* spp. After undergoing dental extraction for caries at age 10 years, he developed respiratory failure and subsequently ventilator-associated pneumonia (with endotracheal tube secretions positive for Methicillin-sensitive *Staphylococcus aureus* and *Moraxella catarrhalis*), as well as *Actinomyces oris*-positive bacteremia. He required a tracheostomy due to ongoing ventilator dependence and critical illness myopathy (biopsy confirmed) and remains ventilator dependent. The patient also has severe global developmental delay, seizures, scoliosis, central sleep apnea, and dysmorphic features, including short/abnormally shaped fingers, hypertelorism, epicanthal folds, long eyelashes, broad nasal base, and microcephaly. Chromosomal microarray showed a normal male genomic profile with multiple regions of homozygosity, but no diagnostic copy number changes (ISCN: arr(1-22)x2,(XY)x1).

The patient's clinical immune phenotype was notable for low CD19⁺ B cells (particularly affecting the isotype-switched IgM⁺IgD⁺ memory B-cell population), low CD4⁺ T cells, increased numbers of CD4⁺CD8[−] (double-negative) TCRαβ⁺ T cells, and impaired T cell proliferation in response to a panel of mitogens including phytohemagglutinin (PHA), concanavalin A (ConA), and pokeweed mitogen (PWM) (Table 1, Table S1). All other tests were unremarkable. Collectively, the history of recurrent infections, abnormal B cell development, and poor lymphocyte proliferation raised the suspicion of an underlying primary immune defect, specifically CID.

Whole exome sequencing of the trio of patient and unaffected parents identified that the patient was homozygous for a *CARD11* variant (NM_032415.5, c.1798G>T, p.Asp600Tyr) that was classified as a variant of unknown significance (Supporting Information). *CARD11* is a highly constrained gene that is extremely intolerant of missense and loss-of-function variation based on the ExAC database (z-score = 4.35 for missense constraint, pLI = 1.00 for loss of function). The *CARD11* D600Y variant was an exceedingly rare variant not found in ClinVar, dbSNP, EVS (<http://evs.gs.washington.edu/EVS/>; accessed on April 2018), or 1000 genome project. It was found once in gnomAD with an allele frequency of 4.08×10^{-6} and twice in ExAC with an allele frequency of 1.687×10^{-5} . Importantly, no individuals homozygous for this variant have ever been reported. Consistent with the marked change in biophysical characteristics at this position caused by the conversion of an aspartic acid (negatively charged) to tyrosine (hydrophobic aromatic), the D600Y variant was predicted to be damaging by SIFT (score = 0.02), CADD (score = 23.7), MutationTaster, Align GVGD (GV = 0.00, GD = 159.94; Class C65), and FATHMM (score = 0.85699), though not by PolyPhen-2 (score = 0.086). Multiple sequence alignment of the substitution site revealed that the D600 amino acid residue had been moderately conserved during evolution (Figure 1A).

Given its crucial role in immunity, the *CARD11* variant was selected for further investigation.³⁻⁵ The single base pair substitution (NM_032415.5:c.1798G>T), leading to an aspartic acid to tyrosine amino acid substitution (NP_115791.3:p.D600Y), was located in repressive element (RE) 3 of the inhibitory domain (ID) of the *CARD11* protein (Figure 1A). Sanger sequencing was performed on both

whole blood and lymphoblastoid B cells (LCLs) from the patient and remaining family members. This confirmed that the parents were heterozygous (I-1, I-2), two siblings were heterozygous (II-3, II-4), and the eldest sister (II-2) was also homozygous for the same substitution (Figure 1A, Figure S1A-B, Supporting Information). This substitution did not render the mRNA subject to nonsense-mediated mRNA decay, nor did it affect the expression of the protein, suggesting that protein stability or dynamics were intact (Figure 1B, Figure S1C, Supporting Information).

As *CARD11* plays a major role in both the assembly of the CBM signalosome and NF-κB activation, we interrogated the effect of the p.D600Y substitution on NF-κB signaling as measured by the phosphorylation of downstream signaling effectors (Supporting Information). LCLs from all members of the family and a healthy control were stimulated for 15 minutes with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μmol/L ionomycin (henceforth referred to as P/I) and immunoblotted. The index patient and his sister with the same genotype (II-2) exhibited patterns of phosphorylation of the p65 subunit of NF-κB, degradation of inhibitor of kappa B (IκBα), and phosphorylation of the IκB kinases (IKK) α/β that were indistinguishable from other family members and a healthy control (Figure 1B). As an internal control to interrogate a pathway not dependent on *CARD11*, we assessed the activation of the mitogen-activated protein kinase (MAPK) pathway⁶ by measuring the phosphorylation of MAPK1/3. As expected, these responses did not differ according to *CARD11* genotype (Figure 1B). Finally, as the phosphorylation of the inhibitory domain of *CARD11* by protein kinase C (PKC) has been shown to regulate NF-κB activation,⁷ the phosphorylation of a serine residue (S652) downstream of the D600Y substitution site was assessed. This modification was also indistinguishable across *CARD11* genotypes, suggesting that the ability of the D600Y variant to be phosphorylated was intact (Figure 1B).

Next, we investigated whether direct activity of CBM assembly was affected in the patient by examining MALT1 paracaspase function. LCLs were stimulated for 4 hour and blotted for the established MALT1 substrate heme-oxidized IRP2 ubiquitin ligase 1 (HOIL1).⁸ The cleavage of HOIL1 was intact in all samples tested, suggesting that MALT1 activity was unaffected by the D600Y *CARD11* variant (Figure 1C). In addition, both phospho-IKKα/β and phospho-MAPK1/3 were comparable to the healthy control, indicating that even at longer time points, the activation of NF-κB and MAPK pathways in patient LCLs was normal (Figure 1C). Downstream target genes also appeared to be unaffected by the *CARD11* genotypes through the measurements of pro-inflammatory cytokine IL-6 secretion (Figure 1D, Supporting Information).

To explore whether there could be kinetic differences in NF-κB activation, LCLs from the two D600Y homozygous subjects (II-1, II-2) and a healthy control were stimulated with P/I across a time course and NF-κB and MAPK activity was measured by immunoblot. Again, regardless of *CARD11* genotype, LCLs derived from these patients demonstrated comparable response kinetics (Figure 1E). Similarly, expanded primary T cells from all family members and a healthy control that were stimulated with P/I for

TABLE 1 Immune profile of the patient

	Patient		Reference Range	
	Abs. #	%	Abs. #	%
Leukocytes	12.6	—	5-11	—
Neutrophils	*9.31	73.9	1.5-8.5	51-82
Monocytes	0.77	*6.1	0.0-0.8	1.0-6.0
Eosinophils	*0.77	*6.1	0-0.5	0.0-3.0
Lymphocytes	1.64	*13.0	1.5-6.0	30-40
CD3 ⁺ mature T cells	*1095	*55	1200-2600	60-76
CD3 ⁺ CD4 ⁺ T helper cells	*279	*14	650-1500	31-47
Naïve CD4 ⁺	—	*32.2	—	33.0-73.5
Memory CD4 ⁺	—	*67.0	—	26.3-66.3
CD4 ⁺ central memory	—	35.1	—	8.9-34.1
CD4 ⁺ effector memory	—	60.3	—	33.4-74.1
CD4 ⁺ TEMRA	—	*1.0	—	0.0-0.8
CD3 ⁺ CD8 ⁺ cytotoxic T cells	637	*57.1	370-1100	18-35
Naïve CD8 ⁺	—	*13.2	—	36.3-83.5
Memory CD8 ⁺	—	*67.1	—	12.9-51.3
CD8 ⁺ central memory	—	*0.2	—	0.6-6.7
CD8 ⁺ effector memory	—	27.8	—	6.6-76.0
CD8 ⁺ TEMRA	—	*19.9	—	0.0-10.3
TCRa/p ⁺ CD4 ⁺ CD8 ⁺ T cells	—	*7.6	—	<2
CD19 ⁺ B Cells	*200	15	270-860	13-27
CD19 ⁺ CD27 ⁺ memory B cells	—	14	—	14-38
IgM ⁺ IgD ⁺ memory	—	12	—	8-26
IgM ⁺ IgD ⁺ (of CD27 ⁺)	—	*85	—	44-72
IgM ⁺ IgD ⁻ memory	—	0.5	—	0.8-4.3
IgM ⁺ IgD ⁻ (of CD27 ⁺)	—	*3.7	—	3.9-16.6
IgM ⁻ IgD ⁻ memory	—	*1.4	—	2.4-9.6
IgM ⁻ IgD ⁻ (of CD27 ⁺)	—	*10	—	16-40
NK cells (CD3 ⁻ CD56 ⁺ CD16 ⁺)	*617	*31	100-480	4-17
Mitogen stimulation	CPM	SI	CPM	SI
PHA	*45 377	*124	93 720	272
ConA	*15 352	*42	99 920	293
PWM	*19 007	*52	97 333	283
Serum immunoglobulin				
IgG (g/L)	14.9	—	5.46-16.18	—
IgA (g/L)	2.9	—	0.54-3.78	—
IgM (g/L)	0.74	—	0.21-2.63	—
IgE (IU/mL)	174	—	<200	—
Specific antibodies (mg/L)				
Measles/mumps/rubella	+/-/+	—	+/-/+	—
α-Tetanus	+ (2.7)	—	+	—
α-Pneumococcus	+ (pre: 6.8; post: 19.6)	—	+	—

Clinical immunophenotyping of patient blood. Values deviating from the reference range are indicated with an asterisk. Shown are the absolute numbers (Abs. #) and or percentages (%) of each population.

TCR, T-cell receptor, NK, natural killer, PHA, phytohemagglutinin, ConA, concanavalin A, PWM, pokeweed mitogen.

0 minutes, 5 minutes, 15 minutes, 30 minutes, and 60 minutes did not show observable differences in both p65 phosphorylation and κ B α degradation, indicating normal lymphocyte response kinetics (Figure S1D, Supporting Information).

CARD11 is involved in signal transduction through a variety of receptors, including the B cell receptor (BCR) and CD40 receptor. Thus, LCLs from the index patient were stimulated with either 10 μ g/mL of a cocktail of anti-IgG+IgM antibodies or 10 μ g/mL of CD40L for 15 minutes and immunoblotted. CD40L stimulation induced robust phosphorylation of p65, degradation of κ B α , and phosphorylation of IKK α / β in both the patient and a healthy control (Figure 1F). However, MAPK1/3 was not activated by CD40L, consistent with previous reports.³ BCR stimulation caused a moderate increase in p65 phosphorylation and κ B α degradation, which was indistinguishable between the patient and healthy control. Finally, to exclude the possibility of artefacts related to overstimulation with P/I, LCLs from the patient, a healthy control, and a MALT1-deficient patient (homozygous p.W580S mutation) known to have defective NF- κ B activation⁸ were stimulated with both half the standard dose ("Low") and standard dose ("High") of P/I and blotted. A dose-dependent effect was seen in all LCLs except those from the MALT1 patient, which exhibited their characteristic diminished response (Figure 1F).

The cost-effectiveness and relative efficiency of WES have led to its increased incorporation into clinical diagnostics, medical genetics, and research. This has greatly empowered the discovery, research, understanding, and treatment of PIDs. However, as many PIDs display genetic heterogeneity (different gene mutations causing similar phenotypes), genetic pleiotropy (different mutations of one gene causing varying clinical/immunologic phenotypes), and incomplete clinical penetrance, it is not possible to rely solely on sequence data to secure a diagnosis. It is essential to establish a causal relationship between genotype and phenotype, especially in the case of novel variants and genes. Even with advances in bioinformatics, no algorithm can definitively state whether a variant is disease causing or not. Thus, it is imperative that rigorous experimental functional characterization of variants be done to avoid assigning a causal role to benign variants.

Here, we present a Canadian First Nations patient (II-1) with recurrent infections and poor lymphocyte proliferation suggestive of CID who was found to be homozygous for a novel p.D600Y CARD11 variant. Although this variant was predicted to be pathogenic by multiple in silico models, it had no functional impact on assays designed to interrogate the function of CARD11 (and the CBM binding partner MALT1). Response patterns of NF- κ B, MAPK, and cytokine secretion in both B and T cells were intact. Consistent with these findings, during the course of these functional experiments, we discovered that one healthy sister (II-2) was also homozygous for this novel CARD11 variant. These results are further supported by the finding that in vitro mutation of RE3 alone in murine CARD11 had no functional impact.⁷

An important consideration in this case is the fact that the patient was from an Indigenous population that may be absent or underrepresented in the population databases used for initial bioinformatics screening of variants. Previous reports have shown that genomics for the purpose of precision medicine fails in diversity,

and certain poorly represented groups are being misdiagnosed.⁹ In Canada, genomic data from Indigenous peoples are limited, and this poses a major public health concern because this impedes diagnosis, prophylaxis, and management of various illnesses, as well as the identification of affected or at-risk relatives. Some genetic conditions found in First Nations individuals may be unique to specific communities, making them extremely challenging to diagnose and treat. This deficit could be ameliorated by recruiting small numbers of these individuals over time, which has been shown to improve diagnostic ability.¹⁰

In this report, we have provided further support for the need to carefully characterize the functional status of variants found by WES, in order to establish a genotype-phenotype relationship. In addition, given the relative lack of diversity in large population databases, especially when it comes to Indigenous populations such as the First Nations, there is a need to increase recruitment of these populations in order to document unique and specific variants that may be common in those communities. These factors will ultimately strengthen the diagnosis and management of PIDs and related diseases.

CONFLICT OF INTEREST

None.

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Keywords

CARD11, caspase recruitment domain family member 11, NF- κ B, primary immunodeficiency, whole-exome sequencing

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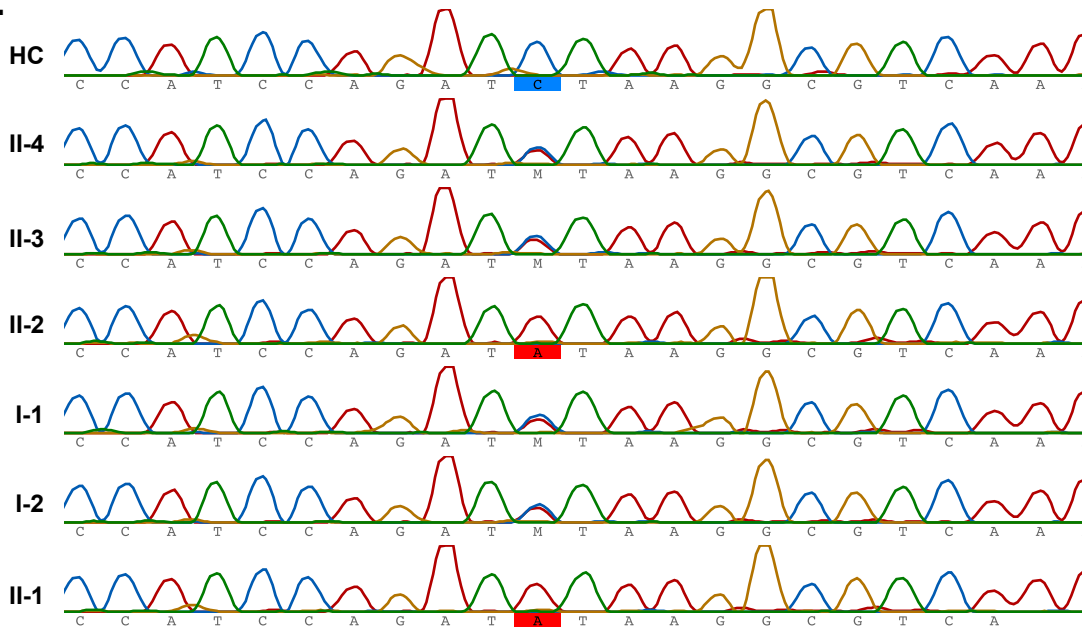
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

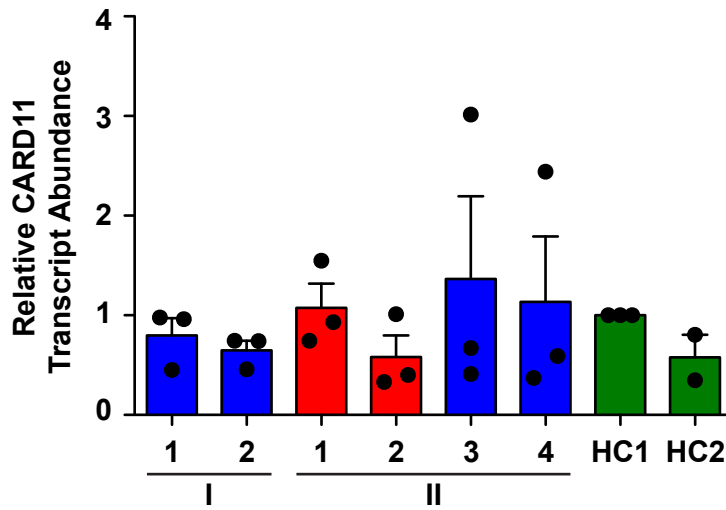
A.

Assay	Gene	Primer (5'-3')	Direction
qPCR	<i>CARD11</i>	GCCGGATGTAGAACGAGTC	F
		CACTACAAGGTCAACCACGAA	R
Genomic sequencing	<i>CARD11</i>	GTCGGAGAAGAGTGAGAGAGA	F
		ATGGTGCCTGGCTAGAGTAT	R
qPCR	<i>ACTB</i>	GTTGCGTTACACCCTTTCTT	F
		ACCTTCACCGTTCCAGTTT	R

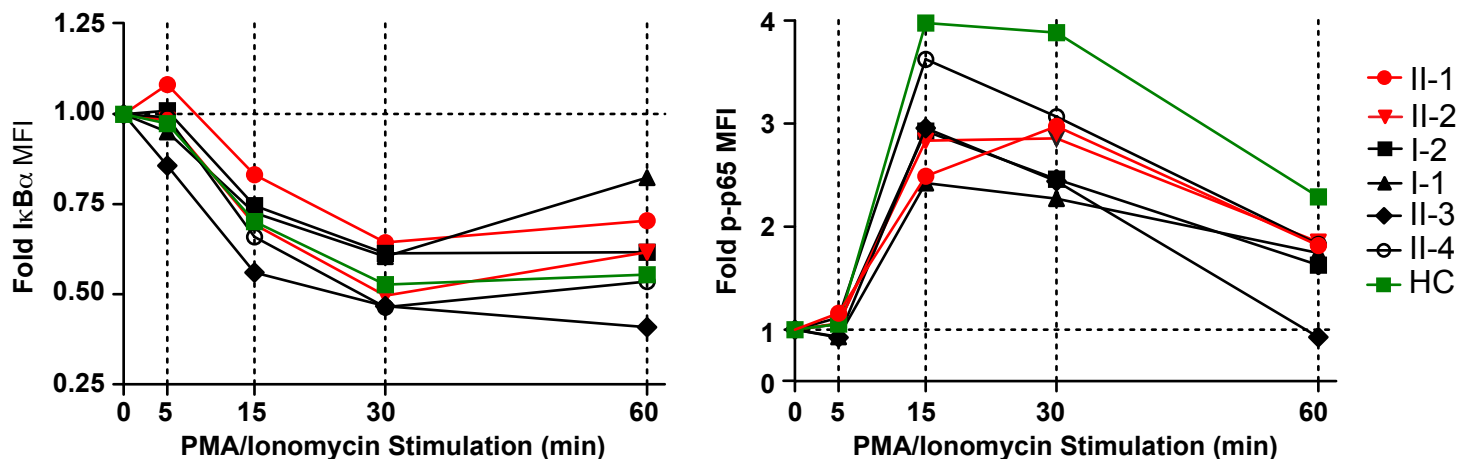
B.



C.



D.



Supporting Information

The importance of functional validation after next generation sequencing: evaluation of a novel *CARD11* variant

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METHODS:

Human blood donors and consent

Research study protocols were approved by The University of British Columbia Research Ethics Board. Six members of the family, including the index patient, parents, three siblings, as well as two healthy controls were enrolled.

Peripheral blood mononuclear cell isolation, B cell immortalization, primary T cell expansion, and cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood by Ficoll-Plaque (GE Healthcare) density centrifugation. Primary immortalized B cells (or lymphoblastoid cell lines [LCLs]) were established by Epstein-Barr virus (EBV) transformation (1). Briefly, fresh PBMCs were treated with Tacrolimus (Prograf) then cultured in complete RPMI-1640 media (GE Healthcare) supplemented with 10% fetal bovine serum (Gibco, Life Technologies), 2mM L-glutamine (HyClone, Fisher Scientific), and 1X Penicillin-Streptomycin (Gibco, Fisher Scientific) along with supernatant from a viral replication-permissive marmoset cell line B95-8 (ATCC) until sufficient B cell blasts were present. Primary T cells were expanded from fresh PBMCs using a T cell activation/expansion kit (#130-091-441, Miltenyi Biotec). T cells were cultured in IMDM (HyClone, Fisher Scientific) supplemented with 10% FBS, 2mM L-glutamine, β -mercaptoethanol, and 1x MEM NEAA (Gibco, Life Technologies) along with beads loaded with anti-CD2, -CD3, and -CD28 antibodies and expanded in recombinant human IL-2 (#200-02, PeproTech).

Immunoblotting

LCLs were stimulated with 50 ng/mL PMA + 1 μ M ionomycin, 25 ng/mL PMA + 0.5 μ M ionomycin, 10 μ g/mL anti-IgG+IgM (#109-005-044, Jackson ImmunoResearch), or 10 μ g/mL CD40L (#3583SC, Cell Signaling Technology). Quantification of protein expression and phosphorylation was done using standard techniques (2). Briefly, patient and family member LCLs were lysed in modified RIPA buffer with Halt protease and phosphatase inhibitors (Thermo Scientific) and proteins were then separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Immobilon-FL, EMD Millipore). Membranes were incubated

for at least 18 h at 4°C with primary antibodies against the following targets: phospho-CARD11 (Ser652; #5189), CARD11 (#4435), phospho-NF-κB p65 (Ser536, #3031), IκBα (#9242), phospho-IKKα/β (Ser176/180; #2697), and β-actin (#3700 or #8457), all from Cell Signaling Technology, phospho-MAPK1/MAPK3 (Thr202/Tyr204; #MA5-15174) from Thermo Scientific, and HOIL-1 (clone 2E2; #MABC576) from Millipore Sigma. Membranes were then incubated with secondary antibodies conjugated with infrared dyes (IRDye 680RD and IRDye 800CW), both from LI-COR Biosciences and imaged on a LI-COR Odyssey infrared imager (LI-COR Biosciences).

Enzyme-linked immunosorbent assays (ELISA)

LCLs were stimulated with 50 ng/mL PMA + 1 μM ionomycin for 4 h. Supernatants were collected and IL-6 was detected by sandwich ELISA (#88-7066-88, ThermoFisher Scientific) (3).

Identification of *CARD11* variant by whole exome sequencing and *in silico* modelling of pathogenicity

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1 **Supporting Information**

2 **The importance of functional validation after next generation sequencing: evaluation of a** 3 **novel *CARD11* variant**

4 *Lu et al., 2018*

5 **METHODS:**

6 Human blood donors and consent

7 Research study protocols were approved by The University of British Columbia Research Ethics
8 Board. Six members of the family, including the index patient, parents, three siblings, as well as
9 two healthy controls were enrolled.

10 Peripheral blood mononuclear cell isolation, B cell immortalization, primary T cell expansion, 11 and cell culture

12 Human peripheral blood mononuclear cells (PBMCs) were isolated from blood by Ficoll-Plaque
13 (GE Healthcare) density centrifugation. Primary immortalized B cells (or lymphoblastoid cell
14 lines [LCLs]) were established by Epstein-Barr virus (EBV) transformation (1). Briefly, fresh
15 PBMCs were treated with Tacrolimus (Prograf) then cultured in complete RPMI-1640 media (GE
16 Healthcare) supplemented with 10% fetal bovine serum (Gibco, Life Technologies), 2mM L-
17 glutamine (HyClone, Fisher Scientific), and 1X Penicillin-Streptomycin (Gibco, Fisher Scientific)
18 along with supernatant from a viral replication-permissive marmoset cell line B95-8 (ATCC) until
19 sufficient B cell blasts were present. Primary T cells were expanded from fresh PBMCs using a
20 T cell activation/expansion kit (#130-091-441, Miltenyi Biotec). T cells were cultured in IMDM
21 (HyClone, Fisher Scientific) supplemented with 10% FBS, 2mM L-glutamine, β -mercaptoethanol,
22 and 1x MEM NEAA (Gibco, Life Technologies) along with beads loaded with anti-CD2, -CD3,
23 and -CD28 antibodies and expanded in recombinant human IL-2 (#200-02, PeproTech).

24 Immunoblotting

25 LCLs were stimulated with 50 ng/mL PMA + 1 μ M ionomycin, 25 ng/mL PMA + 0.5 μ M
26 ionomycin, 10 μ g/mL anti-IgG+IgM (#109-005-044, Jackson ImmunoResearch), or 10 μ g/mL
27 CD40L (#3583SC, Cell Signaling Technology). Quantification of protein expression and
28 phosphorylation was done using standard techniques (2). Briefly, patient and family member
29 LCLs were lysed in modified RIPA buffer with Halt protease and phosphatase inhibitors (Thermo
30 Scientific) and proteins were then separated by 10% SDS-PAGE and transferred onto a
31 polyvinylidene difluoride membrane (Immobilon-FL, EMD Millipore). Membranes were incubated

for at least 18 h at 4°C with primary antibodies against the following targets: phospho-CARD11 (Ser652; #5189), CARD11 (#4435), phospho-NF-κB p65 (Ser536, #3031), IκBα (#9242), phospho-IKKα/β (Ser176/180; #2697), and β-actin (#3700 or #8457), all from Cell Signaling Technology, phospho-MAPK1/MAPK3 (Thr202/Tyr204; #MA5-15174) from Thermo Scientific, and HOIL-1 (clone 2E2; #MABC576) from Millipore Sigma. Membranes were then incubated with secondary antibodies conjugated with infrared dyes (IRDye 680RD and IRDye 800CW), both from LI-COR Biosciences and imaged on a LI-COR Odyssey infrared imager (LI-COR Biosciences).

Enzyme-linked immunosorbent assays (ELISA)

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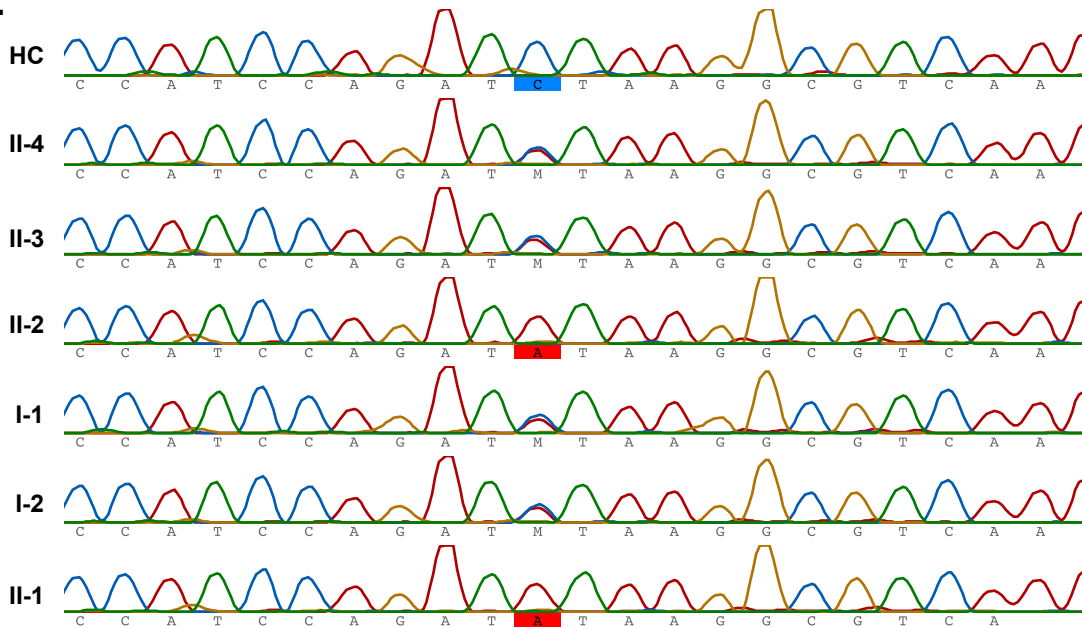
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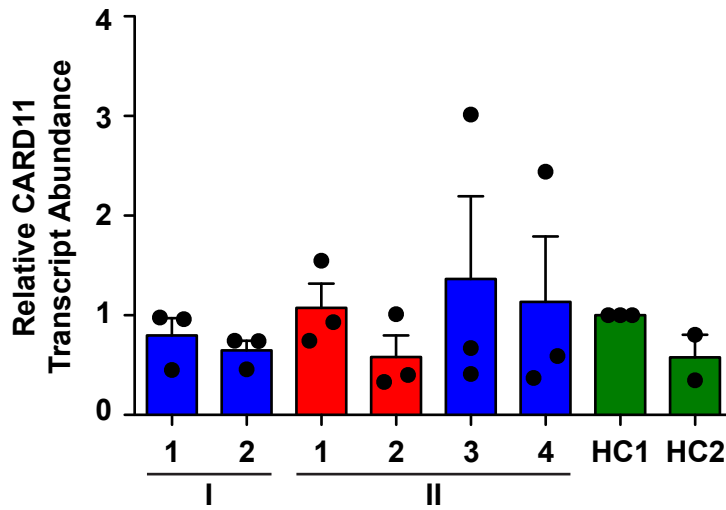
A.

Assay	Gene	Primer (5'-3')	Direction
qPCR	<i>CARD11</i>	GCCGGATGTAGAACGAGTC	F
		CACTACAAGGTCAACCACGAA	R
Genomic sequencing	<i>CARD11</i>	GTCGGAGAAGAGTGAGAGAGA	F
		ATGGTGCCTGGCTAGAGTAT	R
qPCR	<i>ACTB</i>	GTTGCGTTACACCCTTTCTT	F
		ACCTTCACCGTTCCAGTTT	R

B.



C.



D.

