A Unique Heterozygous CARD11 Mutation Combines Pathogenic Features of Both Gain- and Loss-of-Function Patients in a Four-Generation Family

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CARD11 is a lymphocyte-specific scaffold molecule required for proper activation of B- and T-cells in response to antigen. Germline gain-of-function (GOF) mutations in the CARD11 gene cause a unique B cell lymphoproliferative disorder known as B cell Expansion with NF-κB and T cell Anergy (BENTA). In contrast, patients carrying loss-of-function (LOF), dominant negative (DN) CARD11 mutations present with severe atopic disease. Interestingly, both GOF and DN CARD11 variants cause primary immunodeficiency, with recurrent bacterial and viral infections, likely resulting from impaired adaptive immune responses. This report describes a unique four-generation family harboring a novel heterozygous germline indel mutation in CARD11 (c.701-713delinsT), leading to one altered amino acid and a deletion of 4 others (p.His234_Lys238delinsLeu). Strikingly, affected members exhibit both moderate B cell lymphocytosis and atopic dermatitis/allergies. Ectopic expression of this CARD11 variant stimulated constitutive NF-κB activity in T cell lines, similar to other BENTA patient mutations. However, unlike other GOF mutants, this variant significantly impeded the ability of wild-type CARD11 to induce NF-κB activation following antigen receptor ligation. Patient lymphocytes display marked intrinsic defects in B cell differentiation and reduced T cell responsiveness in vitro. Collectively, these data imply that a single heterozygous CARD11 mutation can convey both GOF and DN signaling effects, manifesting in a blended BENTA phenotype with atopic features. Our findings further emphasize the importance of balanced CARD11 signaling for normal immune responses.

Keywords: CARD11, BENTA, Atopy, B cell lymphocytosis, primary immunodeficiencies
INTRODUCTION

BENTA disease [B cell Expansion with nuclear factor kappa B (NF-kB) and T cell Anergy] is a rare immunodeficiency disorder that presents with splenomegaly and unusual peripheral blood lymphocytosis comprised of naïve and immature B cells (1, 2). BENTA is caused by germline, heterozygous GOF mutations in CARD11, which encodes a large multi-domain scaffold molecule best known for connecting antigen receptor (AgR) ligation to NF-κB activation in B and T lymphocytes. Similar to somatic GOF mutations typically found in the coiled-coil (CC) domain of CARD11 and associated with diffuse large B-cell lymphoma (3), BENTA CARD11 mutants spontaneously aggregate to form active signaling clusters with BCL-10 and MALT1 (CBM complex), resulting in constitutive NF-κB activation without requiring B or T cell receptor interaction (4). NF-κB drives the expression of pro-survival genes in transitional and naïve B-cells to confer enhanced survival, while paradoxically rendering T cells less responsive to TCR stimulation with reduced IL-2 production (5–7). Despite the striking expansion of polyclonal B cells, BENTA patients exhibit reduced B cell memory and poor antibody responses to specific vaccines, due in part to intrinsic B cell defects in plasma cell differentiation (8). Moreover, mildly anergic T cell responses may explain increased susceptibility to certain viral infections [e.g., molluscum contagiosum, Epstein-Barr virus (EBV)]. Together, these immune abnormalities increase patients’ susceptibility to viral and bacterial infections, including chronic EBV infection, and may contribute to a higher potential risk of B-cell malignancy (9).

More recently, heterozygous, hypomorphic CARD11 mutations were uncovered in patients with elevated circulating IgE, severe atopic dermatitis, and other allergic manifestations (e.g., rhinitis, asthma, food allergies) (10–12). Strikingly, these variants dominantly interfere with wild-type (WT) CARD11 signaling to both NF-kB and mTORC1, contributing to defective T cell responses that skew toward a T helper 2 (Th2) profile consistent with atopy. Most CARD11 DN patients also share signs of immunodeficiency that overlap with those noted in BENTA patients, including frequent sinopulmonary bacterial infections and viral infections (e.g., molluscum), and fewer class-switched memory B cells. However, B cell lymphocytosis was not observed in this cohort. Furthermore, atopic disease has not been described to date in BENTA patients.

CASE PRESENTATION AND RESULTS

The index patient (IV.1) was referred to our dedicated immunology clinic (McGill University Health Centre-MUHC) at 18 months of age for evaluation of dermatitis and recurrent infections. He was treated with oral antibiotics at 5, 8, and 13 months of age for balanitis. He also suffered from chest X-ray-documented pneumonia and an acute otitis media, which occurred at 6 and 12 months of age, respectively. At 14 months, he was admitted to the hospital for febrile neutropenia with pneumonia and treated with intravenous antibiotics. The neutropenia resolved, but marked persistent lymphocytosis persisted. Small reactive lymphocytes were present on the peripheral blood smear. The physical examination revealed dry lichenified erythematoskin lesions with no palpable lymphadenopathy and no appreciable splenomegaly. Dermatology confirmed a diagnosis of pustular psoriasis, which was complicated by a superimposed recurrent molluscum contagiosum affecting his abdomen, back, perianal and leg areas as well as streptococcal perianal dermatitis.

Initial blood analyses performed at MUHC in 2012 showed a lymphocyte count of 20.4 × 10^9/L and composed predominantly of CD19^+ B-cells (12.4 × 10^9/L, 61.0%), with a decreased proportion of CD3^+ T-cells (6.7 × 10^9/L, 33.0%) and normal CD16^+CD56^+ NK-cells (0.6 × 10^9/L, 3.0%). An extended flow cytometry panel performed in 2015 (MUHC) revealed increased proportions of naïve CD19^+ sIgM^+ B-cells (24.9 × 10^9/L, 72.0%) and CD19^+CD5^+ B-cells (20.0 × 10^9/L, 58.0%). The proportion of class-switched CD19^+CD27^+IgD^- memory B-cells (0.2 × 10^9/L, 0.6%) was decreased. Igh gene rearrangement analysis demonstrated a polyclonal B-cell lymphocytosis pattern (Supplementary Figure 1). The patient had mild hypogammaglobulinemia (IgG 3.1 g/L) associated with slightly decreased serum IgA levels (0.19 g/L), absent serum IgM (<0.25 g/L) and normal IgE levels (63 µg/L). IgE levels increased to 444 µg/L (normal 0–240 µg/L) later when the patient reached 4 years of age. Although, the patient received all recommended vaccines for age (including live attenuated vaccines against measles, mumps, rubella and varicella), he had no specific antibody responses against Haemophilus influenzae type B (IgG <0.1 µg/mL) and Streptococcus pneumoniae (0/14 IgG serotypes were above 1.3 µg/mL). The specific antibody responses were normal for tetanus (IgG above 4.0 IU/mL) and diphtheria toxoids (IgG above 3.0 IU/mL). Lymphocyte proliferation responses to phytohemagglutinin, concanavalin A, and anti-CD3 were also normal. Pokeweed mitogen proliferative response was decreased (stimulation index of 23.2 compared to 85.6 in healthy control). Mild splenomegaly (8.5 cm) was confirmed on abdominal ultrasound. The CT scan showed small bilateral cervical lymph nodes. Blood serology and PCR for EBV and cytomegalovirus (CMV) were negative. The patient was treated with weekly subcutaneous immunoglobulin (scIg) injection therapy (equivalent to 0.65 g/kg/month). At 5 years of age, he suffered from two consecutive pneumonias with the second episode associated with rhinovirus infection, which required hospitalization and oxygen supplementation. Among other relevant medical problems, he is known for mild bilateral conductive hearing loss and atopy IgE-mediated milk allergy (milk specific IgE: 9.95 IU/L and positive skin prick test to cow’s milk extract at 2 years old) and allergic rhinitis to birch and cat.

The family reported that parents were not related and were of Caucasian origin (Figure 1A). The father (Patient III.1, 29 years old) was investigated as a child for splenomegaly, atopic dermatitis, mild intermittent asthma, molluscum contagiosum (on buttocks, legs, and arms from age 1–5 years old) and “high total B-cell number.” No diagnosis was established at the time.

Over the years, he developed recurrent warts and persistent finger onychomycosis (since age 4–5). He had chickenpox at 11 years old followed by shingles as a young adult. More recently, he was treated with intravenous antibiotic for recurrent knee...
effusions and recurrent folliculitis of the forearms and thighs. *Staphylococcus aureus* was isolated on the initial bacterial culture. Further immunological investigations revealed a polyclonal B-cell lymphocytosis (absolute lymphocyte count of $3.4 \times 10^9/L$ with 33.0% of circulating CD19$^+$ B-cells). B-cell subpopulation analysis also demonstrated an increased proportion of naïve CD19$^+\text{slgM}^+$ B-cells ($7.9 \times 10^8/L$, 74.0%) and CD19$^+\text{CD5}^+$ B-cells ($5.3 \times 10^8/L$, 50.0%), while the proportion of class-switched CD19$^+\text{CD27}^+\text{IgD}^-$ memory B-cells ($0.1 \times 10^8/L$, 1.0%) was severely decreased. The father had a mild decrease in serum IgM level (0.48 g/L), but otherwise normal serum immunoglobulins (IgG 12.7 g/L, IgA 1.8 g/L, IgE 37–107 µg/L) and specific antibody responses against tetanus (0.9 IU/mL), diphtheria (0.6 IU/mL), and *Haemophilus influenzae* type B (HIB, 1.9 µg/mL) post vaccination. The specific humoral response against polysaccharide antigens was suboptimal with <50% of pneumococcal serotypes above 1.3 µg/mL despite Pneumovax booster. Numbers and proportions of peripheral blood T and NK cells populations were within normal range for age. Lymphocyte mitogen proliferation assays were also normal. The father was closely monitored for the occurrence of possible lymphoproliferative disorder; he had persistently positive EBV PCR (46 774 copies/mL, EBV VCA positive $\geq 1/4000$, EBV EBNA <1/10) with enlarged axillary lymph nodes (up to 1.7 × 1.2 cm on the left size) and prominent fluorodeoxyglucose activity on PET-CT scan. His spleen size was at the upper limit of normal (13.3 cm). He was treated with frequent boosters of conjugated pneumococcal vaccines (e.g., Prevnar-13) and use of immunoglobulin replacement therapy was considered by the medical team.

The grandmother (II.1) reported frequent otitis externa and colds as a child. As an adult she had two pneumonia and two episodes of shingles, and was treated for Hodgkin’s lymphoma (2A, mixed cellularity) at 33 years old. She has allergic rhinitis to dust and molds, frequent rhinosinusitis, persistent warts, and persistent onychomycosis. The great-grandmother (Patient I.1, 78 years old) suffered from recurrent warts, shingles and sinusitis over the years. A clinical evaluation was offered to the great grandmother at MUHC but never performed. The index patient, father and grandmother were subsequently evaluated at the NIH Clinical Center in 2015 and again in 2017. On physical exam, warts were noted on the index patient (one on toe) and father (multiple on hands). The most recent flow cytometric evaluation of PBMC confirmed expansion of naïve and immature B cells in the index patient, with few class-switched memory B cells detected (Table 1). The same pattern was evident in
TABLE 1 | Flow cytometric phenotyping of patient PBMC and serum Ig levels.

|                | II.1       | III.1      | IV.1       | Normal range (adult) |
|----------------|------------|------------|------------|----------------------|
|                | %          | #          | %          | #                    |
| Total B cells  | 20         | 612        | 32.5       | 1,066                | 43.3       | 1,602                | 3–19                   | 59–329                |
| Naïve CD19+IgD+| 17.7       | 542        | 30.3       | 994                 | 41.3       | 1,528                | 1.4–14.4               | 25–324                |
| CD19+CD10+     | 4          | 122        | 14.5       | 476                 | 33.3       | 1,232                | 0.1–3.4                | 2–76                  |
| CD19+CD27+     | 0.2        | 6          | 0.1        | 3                   | 0          | 0                    | 0.4–2.3                | 5–46                  |
| CD3+ T         | 71.3       | 2,182      | 61.2       | 2,007               | 53         | 1,961                | 60–83.7                | 714–2266              |
| CD4+ T         | 35.1       | 1,074      | 405        | 1,328               | 34.9       | 1,291                | 31.9–62.2              | 359–1566              |
| CD8+ T         | 31.7       | 970        | 16.3       | 535                 | 13.7       | 507                  | 11.2–34.8              | 178–853               |
| NK cells       | 8.9        | 272        | 6.5        | 213                 | 4.1        | 152                  | 6.2–34.8               | 126–729               |
| NKT cells      | 10         | 306        | 6          | 197                 | 3.6        | 133                  | 2.2–12.4               | 29–299                |

Values in blue and red color indicate lower and higher levels, respectively, compared to normal healthy control ranges listed at right. Absolute counts indicated for each cell type are per µL. Normal Ig levels corresponding to patient age groups are indicated in parentheses.

the father and grandmother, although circulating B cell counts decreased inversely with age. PCR indicated moderate EBV viremia in both father and grandmother (3.13 and 4.45 Log10 IU/ml). Serum IgE levels were slightly elevated in the index patient; otherwise total immunoglobulin levels were normal for all subjects. For the father, random antibody titers against rubella, measles, and VZV were normal, but absent against mumps. The grandmother showed normal antibody titers to measles, rubella, mumps, tetanus and HIB, with poor responses to Pneumovax (5/23 serotypes detected).

Affected patients displayed multiple clinical hallmarks of B cell Expansion with NF-κB and T cell Anergy (BENTA) disease, including (a) splenomegaly, (b) polyclonal B cell lymphocytosis, featuring elevated naïve/immature B cells with few class-switched memory B cells, (c) poor responses to pneumococcal vaccines, (d) bacterial/viral infections including pneumonias, molluscum contagiosium/warts, and moderate EBV viremia (2, 9). Because BENTA is caused by gain-of-function (GOF) variants in the lymphocyte scaffold molecule CARD11 (1, 4), we focused on CARD11 as a potential candidate gene. Sanger sequencing was first performed on genomic DNA isolated from peripheral blood mononuclear cells (PBMC) from the index patient (IV.1) and his father (III.1). This analysis revealed a heterozygous four amino acid deletion and missense mutation within exon 5 of CARD11 (NM_032415.3:c.701_713delinsT, p.His234_Lys238delinsLeu, hereafter referred to as H234L/Delta1) expression and harbors an NF-κB-driven green fluorescent protein reporter (GFP) (13). Similar to a confirmed gain-of-function (GOF) CARD11 mutation (E134G) (4), H234L/Delta1-8 induced constitutive NF-κB activation in unstimulated cells, increasing slightly upon TCR/CD28 crosslinking (Figures 2A,B). However, unlike E134G, co-expression of H234L/Delta1-8 with WT CARD11 sharply reduced constitutive NF-κB activity. Strikingly, H234L/Delta1-8 also significantly reduced WT CARD11-dependent NF-κB activation following TCR/CD28 stimulation (Figure 2B). Protein expression of WT, E134G and H234L/Delta1-8 CARD11 were similar in transfected cells (Figure 2C). Co-immunoprecipitations in WT Jurkat transfectants revealed a substantial reduction of total CARD11 association with BCL10 and MALT1 following stimulation in the presence of H234L/Delta1-8 but not WT or E134G CARD11 (Figure 2D). This decrease in CARD11-BCL10-MALT1 (CBM) complex formation was comparable, but slightly less pronounced, than that noted with a bona fide dominant negative (DN) CARD11 variant R47H (11). Moreover, FLAG-tagged CARD11 variants co-precipitated with WT GFP-tagged CARD11 when co-expressed in JPM50.6 cells, indicating WT CARD11 can directly associate with mutant CARD11 protein regardless of stimulation (Figure 2E). Taken together, these results suggest the H234L/Delta1-8 CARD11 mutant can disrupt normal activation-induced CBM complex assembly and NF-κB signaling in T cells through DN interference. Similar to other patients harboring CARD11 DN variants, we also observed marked defects in NF-κB p65 phosphorylation and IκBα degradation in stimulated primary CD4+ T cells from patients III.1 and IV.1 relative to healthy control subjects (Figure 2F).

At baseline, the patient (IV.1) and his father (III.1) showed marked B-cell lymphocytosis: 51.6% and 44.5%, respectively,
of their PBMC were CD19+ B-cells, compared to 13.8% for the patient’s mother (healthy control, HC) and 10.2 ± 4.4% for a reference cohort. The majority were naive IgM+IgD+ B-cells (IV.1 74.9%, III.1 52.1%, HC 8.4%). To assess in vitro B-cell differentiation, PBMC (1 × 10^6/mL) were cultured for 7 days with anti-CD40 (1 µg/mL), IL-4 (200 U/mL), and IL-21 (50 ng/mL) at 1 × 10^6 PBMC/mL in complete medium [RPMI 1640 + 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 15 mM HEPES buffer (pH 7.0), and 100 U/mL penicillin/streptomycin]. IL-21 promotes B-cell differentiation...
impairment in B cell differentiation and T cell hyporesponsiveness to polyclonal stimuli in vitro. (A) PBMCs from mother (healthy control), III.1 and IV.1 were cultured in cRPMI media with anti-CD40 (1 µg/mL) + IL-4 (2000 U/mL) + IL-21 (50 ng/mL) at 37°C and 5% CO2 for 7 days as previously described (6, 15). B cell subpopulations were analyzed by flow cytometry (Becton Dickinson LSR II, FlowJo Software) using Zombie aqua fixable viability dye and the following antibodies: PE-Cy7-conjugated mouse anti-human CD19, APC-conjugated mouse anti-human CD27, FITC-conjugated mouse anti-human CD38, PE-conjugated mouse anti-human CD3, PE-conjugated mouse anti-human IgM. Representative density plots are shown, with gates demarcating short-lived plasma cells (PC) (CD19+ IgM–), class-switched (CS) memory B-cells (CD19+ CD27+ IgM+) and marginal zone B-cells (CD19+CD27+IgM+). (B) Bar graph indicating the percentages of dividing T cells in each group in response to various stimuli. (C) Immunoglobulin production in cell supernatants after 7 days in vitro stimulation of healthy mother and patients’ PBMC with anti-CD40+IL-4+IL-21, quantified by ELISA. Data in (B–C) are representative of three independent experiments. (D) Healthy control, II.1, III.1, and IV.1 patient CD4+ T cells were labeled with 1 µM CFSE and stimulated with 2 µg/mL of soluble anti-CD3 and CD28 Abs ± Protein A (2.5 µg/mL), or MACS iBead particles (1:1 bead:cell ratio) loaded with biotinylated anti-CD2, anti-CD3, and anti-CD28 Abs (Miltenyi Biotec) in cRPMI for 5–6 days. Histogram overlays display T cell proliferation based on CFSE dilution on day 5. (E) Bar graph denoting the percentage of dividing T cells in each group in response to various stimuli. (F) IL-2 levels in day 6 T cell culture supernatants were measured by ELISA as previously described (4, 13). Data in (D–F) are representative of two independent experiments.

**DISCUSSION**

Here we describe a unique four-generation family harboring a novel germline heterozygous indel mutation (c.701–713delinsT) in exon 5 of CARD11, leading to one altered amino acid and a deletion of 3 others (p.His234_Lys238delinsLeu). Clinical and laboratory findings suggested a relatively mild presentation of BENTA disease, based on a modest selective expansion of naïve/immature B cells that inversely correlated with age. Lackluster pneumococcal vaccine responses and moderate EBV viremia were also noted. Also consistent with BENTA disease, our in vitro assays also revealed profound defects in B cell differentiation and Ab secretion, as well as T cell hyporesponsiveness. Interestingly, members of this family also exhibited mild atopic manifestations (e.g., eczema, food allergy, elevated IgE) that waned over time. Collectively, we posit that these effects may result from dual GOF and DN effects of this unique mutation on TCR-induced NF-κB activation (Figure 2). However, in contrast to the family described here, T cells from patients with potent DN mutations generally proliferate poorly even after robust stimulation and strongly skew toward a Th2 phenotype, reflecting more severe defects in TCR-induced NF-κB and
Institutional Review Boards (IRB) of McGill University Health Centre and the National Institutes of Health (NIH). Experiments involving patient blood samples were performed at McGill University Health Center and Uniformed Services University conforming to IRB protocols.

**AUTHOR CONTRIBUTIONS**

MD and SA designed, executed and analyzed patient cell experiments, and wrote the manuscript. JRS, BD, and JS performed transfection experiments. BD performed biochemical and immunoprecipitation experiments. JRS and JN performed Sanger sequencing analysis. MR performed immunoglobulin heavy chain rearrangement analysis. AW, HM, and PA obtained consent and managed clinical care for patients at NIH. MD and BM provided clinical care at MUHC. HS provided clinical care at NIH. HS, BM, and AS supervised research studies and wrote and edited the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02944/full#supplementary-material
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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