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A novel, heterozygous three base-pair deletion in CARD11 results in B cell expansion with NF-κB and T cell anergy disease.

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Abstract

Germline gain-of-function mutations in CARD11 lead to the primary immunodeficiency, B cell expansion with NF-κB and T cell anergy (BENTA). Herein, we report the case of a girl, presenting at 2 years of age with lymphocytosis and splenomegaly in whom a novel, in-frame, three base pair deletion in *CARD11* was identified resulting in the deletion of a single lysine residue (K215del) from the coiled-coil domain. *In vitro* functional assays demonstrated that this variant leads to a subtle increase in baseline NF-κB signaling and impaired proliferative responses following T cell receptor and mitogenic stimulation. Previously reported immunological defects associated with BENTA appear mild in our patient who is now 6 years of age; a B cell lymphocytosis and susceptibility to upper respiratory tract infections persist, however, she has broad, sustained responses to protein-polysaccharide conjugate vaccines and displays normal proliferative responses to *ex vivo* T-cell stimulation.

Case Presentation

A 22 month old girl was referred for assessment of a persistently elevated lymphocyte count identified during the investigation of coryzal symptoms and intermittent diarrhoea. Her symptoms had persisted for over 2 months and culminated in a short hospital admission where she was found to have low-grade pyrexia of 37.8^{o}C, splenomegaly and cervical lymphadenopathy. Total white cell count was elevated at 31.5 x 10^9 cells/L (neutrophils 3.1 x 10^9/L, lymphocytes 26.6 x 10^9/L, monocytes 1.6 x 10^9/L) but C-reactive protein was < 2 mg/L; blood and stool cultures were negative. Symptoms improved following an empirical three day course of azithromycin, however, lymphadenopathy, lymphocytosis and splenomegaly persisted (10.6 cm on ultrasound, upper limit of normal for age: 7 cm).

Past medical history was unremarkable; she had been delivered at term, had no neonatal issues and was growing on the 75th centile for weight. Mild asthma had been treated with a salbutamol inhaler. There was no family history of immunodeficiency or lymphoproliferative disease, however, a maternal great aunt had died of systemic lupus erythematosus. Her parents were not consanguineous.

A summary of immunological investigations is provided in **Table 1**. The CD19^{+} B cell population was expanded at presentation (7.19 x 10^9 cells/L, normal range 0.6-3.1 x 10^9 cells/L), and although it has declined with age, it has consistently accounted for over 50% of the total lymphocyte population (**Figure 1a and 1b**). Longitudinal B cell immunophenotyping **(Figure 1c and Table 2)** has demonstrated expanded populations of naïve B cells with reduced populations of unswitched and switched memory B cells compared to established, age-adjusted reference ranges (1). The B cell population was polyclonal (**Figure 1d**) and a lymph node excision biopsy, performed at 33 months to exclude lymphoproliferative disease in the context of persistent B cell lymphocytosis, was normal.

In light of the B cell lymphocytosis, genetic investigations were undertaken to search for putative gain-of-function mutations in Caspase Recruitment Domain family member 11 (*CARD11)* leading to

the B cell expansion with NF-κB and T cell anergy (BENTA) phenotype. Sanger sequencing revealed a heterozygous, in-frame three base pair deletion that causes the deletion of a single lysine residue at position 215 within the coiled-coil domain of CARD11 (NG_027759.1: c.645-647del; p.Lys215del) (**Figure 2b**). This variant was not identified in the patient's mother; evaluation of the patient's father was not possible (**Figure 2a**). *In silico* analysis of the variant was undertaken: the variant has not been previously reported in the ClinVar, ExAC, gnoMAD or dbSNP databases confirming it was novel. The Mutation Taster score was 1.00, predicting a high probability that the variant is disease causing. PROVEAN analysis (2), an approach that infers the functional consequences of amino acid substitutions and indels, predicted the variant to be deleterious with a score of -14.046. Phylogenetic analysis shows the K215 residue of CARD11 is highly conserved across taxa from human to zebrafish (**Figure 2c**). Other known variants causing the BENTA phenotype are shown in **Figure 2d**. Interestingly, the K215del variant has been reported in the Catalogue of Somatic Mutations in Cancer (COSMIC) (3) as a haematological malignancy-associated somatic mutation in two individuals: one with primary CNS lymphoma (4) and one with chronic lymphocytic leukaemia with Richter's transformation (5).

The functional consequences of the K215del *CARD11* variant on NF-κB signaling were assessed by transfection of CARD11-deficient JPM50.6 cells with mutant and wild-type *CARD11* constructs. Compared to wild-type *CARD11*, the K215del variant showed increased baseline NF-κB signaling, but mildly reduced NF-κB activation following stimulation with anti-CD3/CD28, and substantially reduced activation following stimulation with PMA and ionomycin (**Figure 3a and 3b**). In contrast, the BENTA-associated mutation E134G, increased NF-κB activity following anti-CD3/CD28 ligation. All CARD11 proteins were equally expressed (data not shown). To investigate the effect of the variant in the heterozygous state, K215del *CARD11* was co-transfected in a 1:1 ratio with wild type *CARD11*. Slightly increased baseline NF-κB signaling was again observed, although it did not reach statistical significance. Moreover, modest attenuation of NF-κB signaling in response to both mitogenic and TCR stimulation were again observed. These findings are largely consistent with reported abnormalities in BENTA patients, providing evidence of the functional relevance of this variant in our patient.

The patient, now 6 years old, has been followed up for over 46 months since her original presentation. She continues to suffer from frequent episodes of upper respiratory tract infections

and otitis media that respond well to empirical antibiotic therapy; infection frequency was not reduced by prophylactic antibiotic therapy. There has been a modest reduction in the magnitude of her lymphocytosis over time (**Figure 1a**) and malignant lymphoproliferation and autoimmunity have not emerged. She remains Epstein-Barr virus naïve and continues under close surveillance for future complications of BENTA.

Discussion

CARD11 is a scaffold protein, uniquely expressed in the haemopoetic lineage, responsible for coordinating signaling events downstream of the B and T cell receptor. It is essential for the activation of the canonical NF-κB signaling cascade: following the activation of lymphocyte surface antigen receptors and proximal signaling events, the linker region of CARD11 is phosphorylated by PKC-θ or PKC-β in T cells and B cells, respectively, inducing a conformational change in CARD11 that releases autoinhibition. The subsequent recruitment of BCL10 and MALT1 forms the CBM signalosome complex, and MALT1 recruits TRAF6 to initiate canonical NF-κB signaling (6). CARD11 can also activate the JNK pathway (7) and independently regulates glutamine flux and mTORC1 signaling with consequent effects on Th1/Th17 cell differentiation (8).

Somatically acquired, oncogenic *CARD11* mutations were first identified in tumour samples from individuals with the activated-B-cell subtype of diffuse large B cell lymphoma. The mutations clustered in the CARD11 coiled-coil domain and conferred constitutive activating signals upon the NF-κB pathway (9). CARD11 was subsequently shown to be a critical checkpoint regulating B cell fate following B cell receptor engagement; lymphoma-associated *CARD11* mutations can drive antigen-dependent B cell expansion, providing evidence of synergy between autoreactive B cell receptors and somatic activating mutations within downstream signaling molecules in lymphomagenesis and autoimmunity (10).

The primary immunodeficiency BENTA arises due to germline gain-of-function mutations in *CARD11*. Similar to somatically acquired oncogenic mutations, BENTA-associated mutations tend to cluster in the "LATCH" and coiled-coil domains of CARD11 and constitutively activate NF-κB (11). This leads to a peripheral, polyclonal B cell lymphocytosis, splenomegaly and lymphadenopathy that develops within the first year of life, with the potential to transform into malignant lymphoproliferation (12). The observed B cell lymphocytosis in BENTA is driven by a combination of increased bone marrow output leading to increased transitional B-lymphocytes ($CD10^{+}CD24^{hi}CD38^{hi}$) and enhanced survival in peripheral lymphoid tissue leading to expansion of the naïve B cell compartment. Functionally, naïve B cells from BENTA patients proliferate normally to mitogenic stimuli and show enhanced viability *in vitro,* which contributes to their persistence (12, 13). Despite this, the efficient generation of short-lived plasmablasts and long-lived plasma

cells can be impaired in BENTA due to poor induction of key differentiation factors including BLIMP-1 and XBP-1 (14). Consequently, recurrent sinopulmonary bacterial infections and upper respiratory tract infections are common in BENTA, as is a failure to respond to polysaccharide vaccination and, in some patients, protein-conjugate vaccination (12, 15). Our patient's burden of infectious disease remains modest, restricted to the upper respiratory tract, and she has mounted robust and sustained responses to all vaccines administered routinely as part of the UK vaccine schedule including the 13-valent pneumococcal conjugate vaccine. Autoimmunity may also complicate BENTA less frequently; haemolytic anaemias (16) have been noted, but these have not occurred in our patient.

Constitutive increased CARD11 activity downstream of the T cell receptor (TCR) does not lead to a numerical expansion of T cells, but induces a degree of hyporesponsiveness that can be rescued using very strong stimuli (12). Accordingly, our patient displays normal *ex vivo* proliferative responses to bead-conjugated anti-CD2, anti-CD3 and anti-CD28 stimulation. The T cell defect in BENTA is believed to underlie a susceptibility to complications of EBV infection. Low level chronic EBV viraemia emerges in over 80% of BENTA patients with serological evidence of EBV exposure and is hypothesized to arise secondary to poor TCR induced IL-2 secretion, consequent NK cell dysfunction and an expanded pool of naïve B cells that can maintain lytic infection (13). There may be a wider susceptibility to viral infections in some patients: molluscum and BK virus infection have also been reported (12). To date, our patient remains EBV naïve.

Herein, we report the case of a 6 year-old girl with a BENTA phenotype driven by the deletion of the lysine 215 residue from the coiled-coil domain of CARD11. We confirm this variant leads to both modest enhancement of baseline NF-κB signaling and attenuation of T cell responses to mitogenic and TCR stimuli, consistent with a milder phenotype. The relationship between *CARD11* genotypes, the structural biology of mutant CARD11 within the CBM signalosome complex, the potency of the gain-of-function effect and final immunological and clinical phenotype requires further exploration. For example, contrasting clinical outcomes have been reported for patients possessing the C49Y variant ranging from mild disease to fatal haemophagocytic lymphohistiocytosis (16, 17). A distinct mutation in the coiled-coil (p.His234_Lys238delinsLeu) produced a "mixed" clinical BENTA phenotype with atopic disease features, consistent with both

gain-of-function and dominant negative signaling effects (18). Longitudinal studies of patients with *CARD11* mutations are necessary to fully inform our understanding of this rare disease.

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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Figure legends

Figure 1 - Immunophenotyping of peripheral blood lymphocytes: (A) Longitudinal analysis of the total peripheral blood lymphocyte count, total B cell count and (B) relative size of the $CD19^+$ and $CD3^+$ lymphocyte populations from 26 months of age to present. (C) Flow cytometric analysis of PBMC performed at 69 months of age demonstrating an expanded population of naïve B lymphocytes ($CD19^+$, IgD^+, $CD27^-$), reduced populations of unswitched ($CD19^+$, IgD^+, $CD27^+$) and switched ($CD19^+$, IgD^-, $CD27^+$) memory B cells and increased transitional B cells ($CD19^+$, IgM^{hi}, $CD38^{hi}$) compared to established, age-adjusted reference ranges (1). (D) Flow cytometric analysis performed at 26 months of age demonstrating polyclonal B cell kappa/lambda usage and normal CD4/CD8 populations

Figure 2 – Pedigree and sequencing of c.645-647del mutation in CARD11: (A) Pedigree of kindred. (B) Sanger sequencing chromatograms demonstrating c.645-647del in-frame 3 bp deletion in proband. NG_027759.1 is the reference sequence. (C) Protein alignment of CARD11 K215 demonstrating conservation across taxa. The conserved lysine residue is highlighted in red. Numbers represent the position of the lysine residue relative to N-terminus in each species. (D) Schematic representation of CARD11 protein showing locations of known CARD11 mutations resulting in BENTA (blue) and the deletion of lysine 215 found in the proband (red).

Figure 3 – *In vitro* functional validation of the K215del variant: CARD11-deficient Jurkat T cells containing an NF-κB-driven GFP reporter (JPM50.6) were transfected with mutant or wild-type CARD11 (A and B) or mutant and wild-type CARD11 in a 1:1 ratio (C and D). Transfected cells were stimulated with anti-CD3/anti-CD28 or phorbol 12-myristate 13-acetate (PMA) and ionomycin. The E134G variant is a known BENTA-causing variant and serves a positive control. Representative histograms of NF-κB reporter activity (A and C; numbers denote % GFP+ cells) and GFP mean fluorescence intensity (B and D) are shown (n = 3). Statistical significance was determined using a

one-way ANOVA comparing each variant to empty vector/empty vector plus wild-type for a given treatment condition (*denotes $p < 0.05$ after adjustment for multiple comparisons with Dunnett's test).